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13. ABSTRACT (Maximum 200 Words)
Genes, expression of which is breast specific or is altered during breast tumorigenesis, represent potential targets for new preventive and curative strategies. Such genes, Mammaglobin (MGB1), hSBEM (Human Small Breast Epithelial Mucin), Psoriasin, Estrogen receptor beta (hERB) and SRA (Steroid receptor RNA activator), are currently studied in our group. We found that:

MGB1 expression correlates with ER (an established breast cancer prognostic and predictive factor), but is not modified by hormone treatments, suggesting it might be a new independent breast cancer prognostic marker.

hSBEM, which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics can now be detected using specific antibodies.

SRA, previously believed to believed to belong to the family of non-coding RNA and able to activate steroid receptor activity, is translated into a protein which function remains to be determined.

Psoriansin, highly expressed in high risk DCIS, can contributes to breast tumor progression through modulation of Jab1 activity.

hERB isoforms have promoter-specific differential activities suggesting that they may have a role in differentially modulating estrogen action during breast tumor progression.

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INTRODUCTION

Breast cancer remains one of the most frequently diagnosed cancers today. One in eight women is expected to present with breast cancer within her lifetime in developed countries. An estimated 1,000,000 cases are detected each year worldwide and in Canada alone, an estimated 21,200 women will be diagnosed with breast cancer and 5,300 will be lost to this disease in 2003 (1). For women with recurrent disease, the median time of survival is about two years (2). Despite such striking statistics, breast cancer related mortality is slowly decreasing as continuing research has led to earlier detection, more treatment options for breast cancer patients and an improved chance of long-term survival. Improving the diagnosis and clinical management of breast cancer requires access to and characterization of biomarkers that are able to reflect the molecular phenotype of breast tissue. Genes, expression of which is breast specific or is altered during breast tumorigenesis, represent potential targets for new preventive and curative strategies. Such genes, Mammaglobin (**MGB1**), **hSBEM** (Human Small Breast Epithelial Mucin), **Psoriasin**, Estrogen receptor beta (**hERB**) and **SRA** (Steroid receptor RNA activator) are currently studied in our group.

BODY

MGB1: Mammaglobin A was first identified in 1996, as a breast specific member of the uteroglobin gene family overexpressed in some breast tumors (3, 4). As shown in **APPENDIX 1**, we evaluated MGB1 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively (5). Both MGB1 mRNA and protein expression were significantly higher in estrogen receptor positive compared to estrogen negative tumors (Mann-Whitney rank sum test, $p = 0.04$; Chi-square test, $p = 0.01$; respectively). In contrast, MGB1 expression did not correlate with progesterone receptor levels or Notthingham grade. As estrogen and antiestrogen treatment of estrogen positive breast cancer cell lines does not modify MGB1 expression we suggest that MGB1 could be a new independent breast cancer prognostic marker.

hSBEM: We reported last year the identification of a novel putative breast-specific gene (hSBEM, Human Small Breast Epithelial Mucin), which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics (6, **APPENDIX 2**). We have now obtained mammary cells MCF-7 stably expressing V5-tagged hSBEM protein as well as antibodies recognizing the transfected as well as the endogenous protein. We have just initiated a new project, funded by the CIHR and abstract of which is included in **APPENDIX 3**, to further explore the potential of this protein as a breast cancer biomarker.

Psoriasin: We have previously identified psoriasin (S100A7) as a differentially expressed gene between DCIS and invasive breast carcinoma (7). In collaboration with Dr. Watson, we have found that psoriasin physically interacts with Jab1 (c-jun activation-domain binding protein 1) and we suggest that intracellular psoriasin influences breast cancer progression through stimulation of Jab1 activity (8, **APPENDIX 4**).

hERB: Estrogens regulate the growth and development of normal human mammary tissue and are also involved in breast tumor progression. Estrogen action is mediated mainly through two estrogen receptors (ERs): ER-alpha (9) and ER-ß1 (10, 11). Several variant forms of ER-ß1 have been identified in breast tissues (for reviews see Refs. 12-15). In collaboration with Dr. Murphy, we investigated the putative functional characteristics of human receptor beta isoforms (16, **APPENDIX 5**). We showed that only ER- ß1 was able to bind ligand whereas all ER-beta isoforms bind to DNA even though their binding abilities differ. ER-beta isoforms inhibition of ER-alpha and ER- ß1 transcriptional activity is promoter specific. Overall, our data suggest that ER-beta isoforms may have a differentially modulating estrogen action. As shown in **APPENDIX 6** we also found that the expression of hERB isoforms differ in primary tumors of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy suggesting a possible role of ERB related protein in hormone resistance (17).

SRA: SRA is a steroid receptor co-activator acting as a functional RNA and is classified as belonging to the growing family of functional non-coding RNAs (18). None of the different SRA transcripts described to date encode a detectable SRA protein following in vitro and in vivo translation experiments. We have previously shown that SRA RNA was differentially expressed in normal and tumor breast tissues (19, 20). We have identified three new SRA-RNA isoforms differing mainly from the originally cloned SRA by an extended 5(') extremity. These long SRA isoforms, able to encode a stable protein in vitro, led to the production in vivo of a nuclear protein when transfected into the MCF-7 human breast cancer cell line. Reverse-transcription polymerase chain reaction and Western blot analysis of RNA and protein extracts from different breast cancer cell lines confirmed the presence of endogenous coding SRA isoforms and their corresponding proteins. Our results demonstrate that full-length SRA-RNAs likely to encode stable proteins are widely expressed in breast cancer cell lines (21, **APPENDIX 7**).

KEY RESEARCH ACCOMPLISHMENTS

- We hypothesized that MGB1 could be a newindependent prognostic marker of breast cancer.
- We obtained breast cancer cells stably expressing tagged hSBEM protein.
- We obtained antibodies recognizing both transfected and endogenous hSBEM protein.
- We established a possible mechanism of action for psoriasin in breast tissues.
- We confirmed that hERB isoforms are likely to play a role in response to hormone therapy.
- We have identified new SRA isoforms encoding stable SRA proteins.
- We obtained breast cancer cells stably expressing tagged SRA proteins.
- We obtained antibodies recognizing transfected and endogenous SRA proteins

REPORTABLE OUTCOMES

* Five articles published (APPENDICES 1, 4, 5, 6, 7)

CONCLUSION

Two projects are currently funded within the laboratory: hSBEM and SRA. We are now characterizing the different transfected cell lines and investigating the possible use of SBEM to detect in vivo breast cancer cells and the putative function of SRA protein.

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Appendices

APPENDIX 1: Endocrine. 2003. 21: 00-00

Endocrine, vol. 21, no. 3, 000–000, August 2003 0969–711X/03/21:000–000/$25.00

Relationship Between Mammaglobin Expression and Estrogen Receptor Status In Breast Tumors

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Mammaglobin (SCGB2A2) is a breast-specific member of the secretoglobin (SCGB) gene family. SCGB2A2 has previously been found overexpressed in breast tumors but possible associations between its expression and established prognostic tumor characteristics such as the levels of estrogen and progesterone receptors have not yet been investigated. We evaluated SCGB2A2 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively. Both SCGB2A2 mRNA and protein expression were significantly higher in estrogen-receptor-positive compared to estrogen-receptor-negative tumors (Mann–Whitney rank sum test, $p = 0.04$; chi-square test, $p = 0.01$; respectively). In contrast, SCGB2A2 expression did not correlate with progesterone receptor levels or Notthingham grade. As estrogen and antiestrogen treatment of estrogen-positive breast cancer cell lines does not modify SCGB2A2 expression we suggest that SCGB2A2 may be a new independent breast cancer prognostic marker.

Key Words: SCGB2A2; MGB1; estrogen receptor; progesterone receptor; Notthingham grade; breast cancer.

Introduction

Mammaglobin (MGB1, SCGB2A2) was first identified in 1996, using differential display analysis, as a breast-specific member of the secretoglobin (SCGB) gene family overexpressed in some breast tumors *(1,2)*. Today, a search for breast-specific expressed sequence tags (ESTs) performed using the Differential Gene Expression Displayer (DGED) tool at the Cancer Gene Anatomy Project (CGAP) website (http://cgap.nci.nih.gov/Tissues/GXS) shows that SCGB2A2-related ESTs have been found in nine different breast cDNA libraries but only two non-breast libraries, further confirming the relative breast specificity of SCGB2A2 expression. Using a subtractive hybridization approach, we previously identified SCGB2A2 mRNA as overexpressed in the *in situ* compared to the invasive element within an individual breast tumor *(3,4)*. Further *in situ* hybridization analysis, performed in breast tumors selected to include normal, *in situ*, and invasive primary tumor elements revealed that SCGB2A2 expression, restricted to epithelial cells, could be detected in all elements and was significantly increased in tumor cells compared to normal cells *(4)*. This higher SCGB2A2 expression in malignant versus nonmalignant breast epithelium has also been confirmed at the protein level by immunocytochemistry *(5)*. In this latter study, Watson et al. concluded that SCGB2A2 expression was independent of tumor grade and histological type.

It has recently been demonstrated that circulating mammary carcinoma cells can also be detected in the blood of breast cancer patients via PCR detection of SCGB2A2 mRNA *(6–9)*. Even though its biological function remains unknown, SCGB2A2 is now considered as a relatively specific marker of axillary lymph node breast metastases as well as of occult breast cancer *(10–13)*. Interestingly, Zach et al. detected SCGB2A2 mRNA expression by nested reverse-transcription PCR (RT-PCR) more frequently in the blood of patients with estrogen-receptor-positive (ER+) breast tumor than in the blood of estrogen-receptor-negative (ER–) breast cancer patients, suggesting a possible relationship between SCGB2A2 and ER levels in primary breast tumors *(6)*. In order to investigate further possible associations between SCGB2A2 expression and estrogen and progesterone receptors in primary breast tumors, we assessed SCGB2A2 expression at the mRNA and at the protein level in a cohort of breast tumors.

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Results

Assessment of SCGB2A2 mRNA Expression in a Cohort of 52 Human Breast Tumor Samples

To establish whether SCGB2A2 mRNA expression paralleled established known prognostic parameters such as ER and PR levels, a cohort of 52 cases was selected from the

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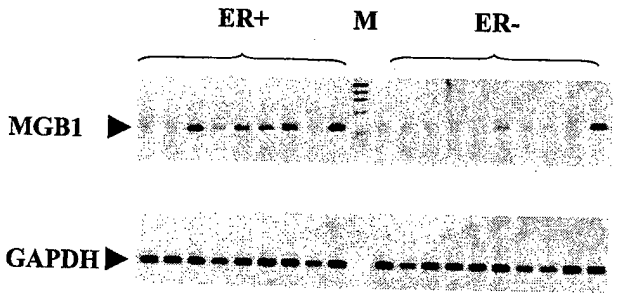

Fig. 1. RT-PCR analysis of SCGB2A2 and GAPDH mRNA expression in primary breast tumors. Total RNA was extracted from frozen tissue sections corresponding to ER positive (ER+) and ER negative (ER–) cases, reverse-transcribed and PCR amplified as described in the Materials and Methods section using SCGB2A2- or GAPDH-specific primers. PCR products were then separated on 2% agarose gels prestained with ethidium bromide. Black arrow: product corresponding to SCGB2A2, grey arrow: product corresponding to GAPDH. M: Molecular weight marker (Φx174 RF DNA/*Hae*III fragments, Gibco BRL, Grand Island, NY).

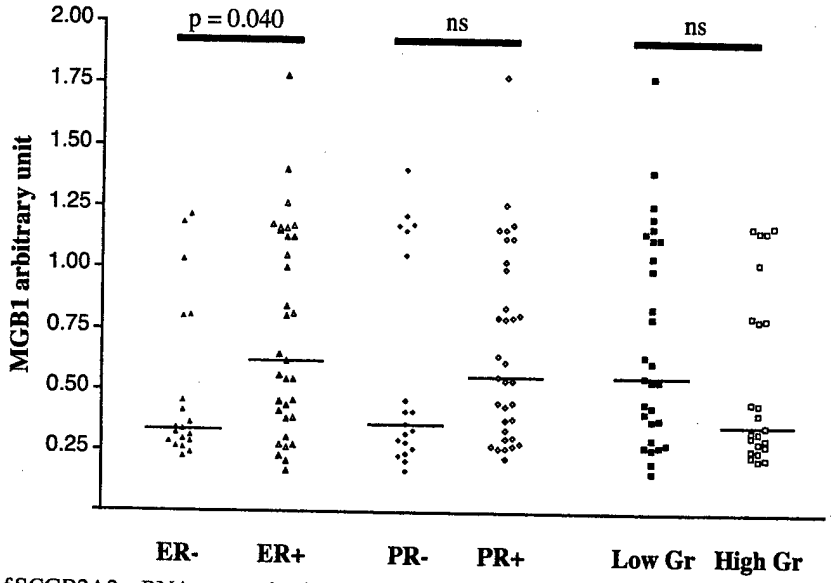

Fig. 2. Quantification of SCGB2A2 mRNA expression in different breast tumor subgroups. Total RNA was extracted from frozen tissue sections corresponding to 52 cases and analyzed as described in Fig. 1. SCGB2A2 mRNA expression was quantified relative to GAPDH mRNA as described in the Materials and Methods section. Tumors were grouped according to their ER status (ER+, ER–), their PR status (PR+, PR–) or their grade (Low Gr: Notthingham scores between 5 and 7; High Gr: Notthingham scores between 8 and 9). Difference between subgroups were tested using the Mann–Whitney rank sum test, two-sided.

NCIC-Manitoba Breast Tumor Bank. For each case, clinical characteristics of the tumor (i.e., ER and PR levels, Nottingham grade) were known (*see* Materials and Methods for a summary of tumor subgroup characteristics). Total RNA was extracted from frozen primary tumor sections, reverse-transcribed and analyzed by RT-PCR using primers recognizing specifically SCGB2A2 cDNA, and chosen to span intronic regions. As shown Fig. 1, SCGB2A2 corresponding signal can be detected in the majority of cases, even though levels of expression varied from one sample to another. Amplification of the ubiquitously expressed GAPDH cDNA in the same cDNA samples was performed in parallel and, for each case, a normalized SCGB2A2 mRNA expression value was calculated (*see* Materials and Methods). SCGB2A2 expression was found to strongly correlate with ER levels ($n = 52$, Spearmann coefficient $r = 0.282$, $p = 0.042$) but not with PR levels or grade (data not shown). Similarly (Fig. 2), using the established clinical cut-off of ER positivity (ER positive tumors have a binding higher than 3 fmol/mg of total protein), SCGB2A2 mRNA expression was significantly (Mann–Whitney rank sum test, two-sided, $p = 0.040$) higher in ER+ ($n = 33$, median value SCGB2A2 = 0.62) than in ER– ($n = 19$, median SCGB2A2 value = 0.33).

Assessment of SCGB2A2 Protein Expression in a Cohort of 32 Human Breast Tumor Samples

In order to determine whether SCGB2A2 protein expression correlated with SCGB2A2 mRNA expression and whether a similar association between ER status and SCGB2A2 expression could be observed at the protein level, paraffin

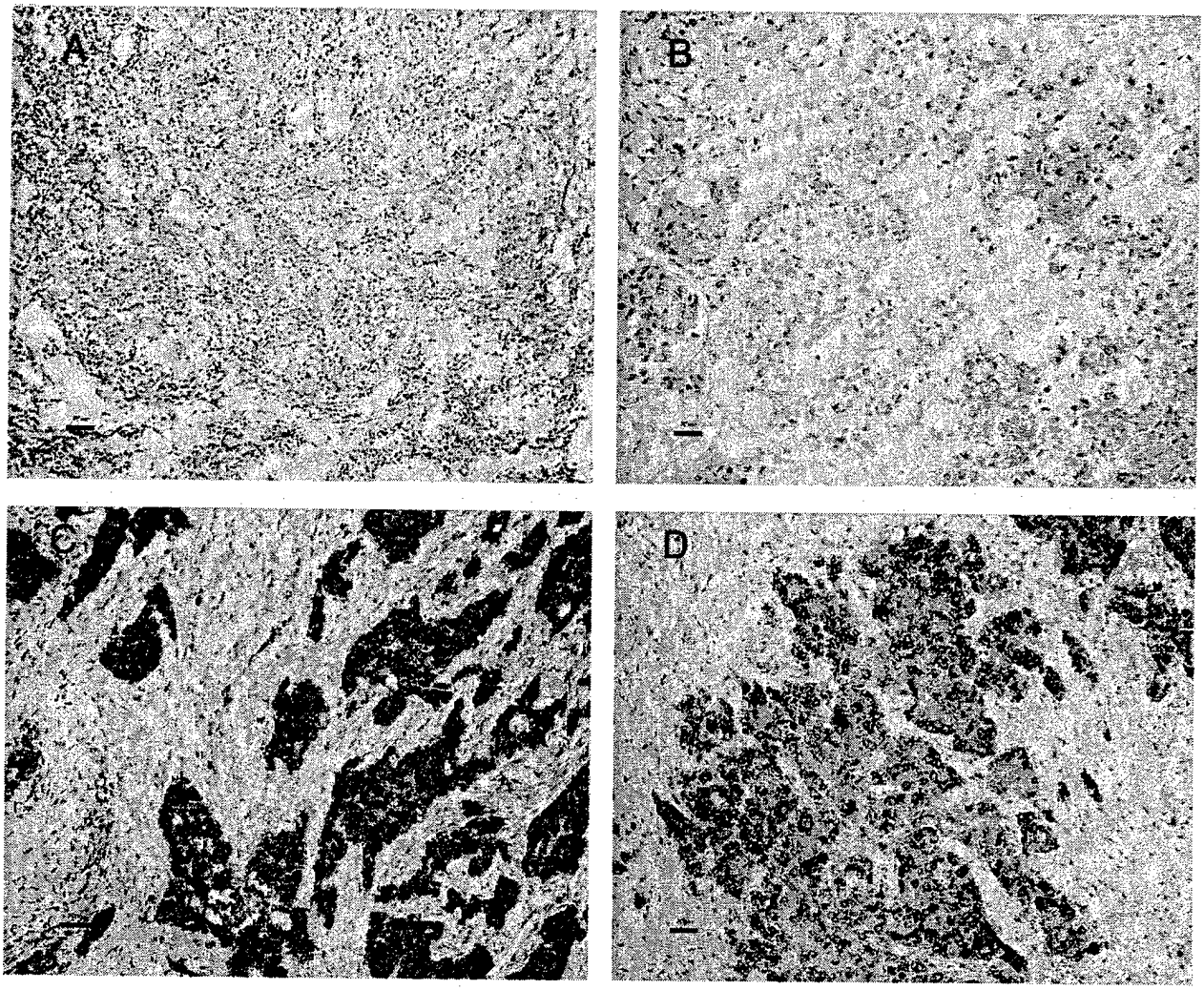

Fig. 3. Detection of SCGB2A2 protein in breast tumors by immunohistochemistry. SCGB2A2 protein was detected on paraffin-embedded breast tumor tissue sections using a rabbit polyclonal primary anti-SCGB2A2 antibody (Kindly provided by Dr. T Fleming) and the Ventana-Discovery system as described in the Materials and Methods section. Panel A and B: Two independent ER– cases showing no (A) or low (B) SCGB2A2 protein expression in tumor epithelial cells. Panel C and D: two independent ER+ cases presenting a strong SCGB2A2 signal. Blue bar: 20 μm.

blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression (*see* Materials and Methods). Slides were scored blindly for SCGB2A2 protein expression by a pathologist as described in the Materials and Methods section. Some sections showed no (Fig. 3A, SCGB2A2 score = 0) or low (Fig. 3B, SCGB2A2 score = 1) SCGB2A2 expression, whereas others presented strong SCGB2A2 protein signal (Fig. 3C, SCGB2A2 score = 3; Fig. 3D, SCGB2A2 score = 2). Comparison of SCGB2A2 protein scores and previously obtained normalized SCGB2A2 mRNA levels revealed a strong correlation ($n = 32$, Spearman r coefficient $r = 0.575$, $p = 0.0006$) between protein and mRNA levels. Tumors were classified as low (scores between 0 and 1) and high (1.5 and 3) SCGB2A2 protein expressors, and differences between tumor subgroups (ER+/ER–, PR+/PR–, low grade/high grade) were assessed using chi-square test. As observed for SCGB2A2 mRNA, SCGB2A2 protein positivity was associated (chi-square test, $p = 0.017$) with ER status but not with PR status or grade (Fig. 4).

Absence of Estrogen Regulation of SCGB2A2 Expression

These data suggested that estrogen might regulate SCGB2A2 expression. In order to address the question of a possible regulation of SCGB2A2 expression in breast cancer cells, ZR-75 cells, known to express SCGB2A2 *(14)*, were treated by estradiol-17β 10^{-8} *M* or the antiestrogen ICI-182,780 10^{-6} *M* for 6, 24, and 48 h as described in the Materials and Methods section. Total RNA was extracted and analyzed by RT-PCR using primers recognizing GAPDH, SCGB2A2, or psoria-

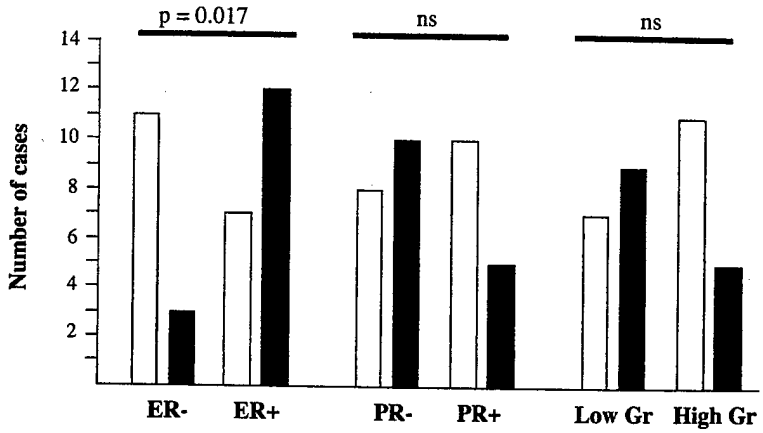

Fig. 4. Quantification of SCGB2A2 protein in breast tumor subgroups. Paraffin-embedded tissue section corresponding to 32 cases were processed as shown Fig. 3. Slides were independently reviewed and scored as described in the Materials and Methods section. For each tumor subgroup (ER–, ER+, PR+, PR–, low grade, and high grade), the number of cases negative (White columns) or positive (black columns) is shown. Differences between subgroups were tested using the chi-square test.

sin cDNAs. Psoriasin was chosen as its expression has previously been shown to be regulated by estrogen treatment (*15*, our unpublished data). SCGB2A2 mRNA expression was not changed under any treatment condition (data not shown), whereas, as expected, psoriasin signal was found to be increased by estradiol and decreased by antiestrogen treatment as soon as 6 h of treatment, with a maximum effect after 24 and 48 h of treatment (estradiol treatment: 1.5-, 2.8-, and 4.5-fold control and antiestrogen treatment 0.90-, 0.80-, and 0.70-fold control, respectively).

Discussion

Assessment of SCGB2A2 expression at the mRNA and the protein levels in a cohort of breast tissue samples showed a statistically significant relationship between SCGB2A2 levels and ER status. However, within the same cohort, no association was found between SCGB2A2 expression and other known prognostic marker such as PR levels or Notthingham grade.

To the clinician, a factor is considered a prognostic factor when it is associated with the outcome of the disease, i.e., predicts how the disease would evolve if not treated, whereas a predictive factor is associated to the degree of response to therapy, i.e., predicts the likelihood of response to a particular treatment. A high level of ER in tumor tissue has a good prognostic value and also predicts a good likelihood of responding to hormonal adjuvant therapy such as tamoxifen *(16,17)*. As PR expression is positively regulated by estrogens, higher PR levels in ER+ tumors support the hypothesis of an operational ER signaling pathway and is therefore also considered as a good prognostic and predictive parameter. Whereas the parallel between SCGB2A2 and ER expression suggested that SCGB2A2 could be a new ER target gene, the lack of association with a known regulated gene such as PR suggested that SCGB2A2 expression was independent of ER signaling pathway. This latter hypothesis was further supported by the absence of estrogen and antiestrogen regulation of SCGB2A2 expression in ZR-75 cells, even though ER signaling pathway appears functional, as shown by the induction of a known ER-regulated gene, psoriasin. It should be noted that a similar absence of regulation was also observed in another ER+ breast cancer cell line MCF-7 cells (our unpublished results; *18*). However, even though the SCGB2A2 gene was not grossly rearranged in MCF-7 cells *(18)*, these cells do not express endogenous SCGB2A2 (our unpublished results; 2). It might therefore be hypothesized that SCGB2A2 expression in MCF-7 cells is negatively regulated by other factors, resulting in an absence of estrogen regulation in these cells. Further experiments performed on other breast cancer cell lines and primary cells *(19)* are needed to confirm these preliminary results.

Interestingly, the general expression of SCGB2A2 as well its association with ER levels observed in vivo in breast tissue contrasts with in vitro observations made on mammary epithelial cancer cell lines. Indeed, looking at a panel of different breast cancer cell lines, Watson et al. reported the detection of SCGB2A2 transcripts only in few cell lines (MB361, MB415, MB468, BT474, MB175) with no expression in MCF7, MB134, MB231, or MCF10A cells *(2)*. Similarly, we did not detect SCGB2A2 expression in breast cell lines such as BT20, T47D, or MCF10AT1 even though a strong signal was seen in ZR-75 (our unpublished observation). As cells such as MB468 and MB361 are ER– and cells such as ZR-75 or BT474 are ER+, SCGB2A2 expression does not appear related to ER status in cells grown in vitro. Overall, this suggests that most of cell lines, through

selection, medium conditions, and/or dedifferentiation lost their ability to express SCGB2A2 in vitro. Presently, no data are available regarding the possible biological function of SCGB2A2. It has however recently been reported that SCGB2A2 existed in a tetrameric complex with BU101 (lipophilin B), another member of the secretoglobin family, the expression of which correlated with SCGB2A2 expression in breast tissue *(20)*. The role of this complex as well as the possible regulation of its components remains to be determined.

In conclusion, we found that SCGB2A2 expression correlated with ER levels in breast tumor tissue. As ER is considered as a good prognostic factor and as SCGB2A2 does not appear to be directly regulated by the ER signaling pathway, we hypothesize that SCGB2A2 expression may be a new independent prognostic marker in breast cancer. Further experiments performed on a larger cohort of patients and completed with follow up studies are needed to test this hypothesis.

Materials and Methods

Human Breast Tissues and Cell Lines

All breast tumor cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As it has been previously described *(21)*, tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in hematoxylin and eosin (H&E)-stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks. Fifty two tumors were selected, spanning a wide range of estrogen and progesterone receptor levels, as determined by ligand binding assay. Within these tumors, 9 were ER–/PR– (ER < 3 fmol/mg total protein; PR < 10 fmol/mg), 10 were ER–/PR+ (ER < 3 fmol/mg; PR > 10 fmol/mg), 10 were ER+/PR– (ER > 3 fmol/mg; PR < 10 fmol/mg), and 23 were ER+/PR+ (ER > 3 fmol/mg, PR > 10 fmol/mg). These tumors also spanned a wide range of Nottingham grade for ER– ($n = 19$, grade ranging from 5 to 9, median 8) and ER+ ($n = 33$, grade ranging from 5 to 9, median 6) tumors. SCGB2A2 mRNA expression was assessed by RT-PCR on total RNA extracted from frozen tissue sections. Paraffin blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression.

ZR-75 cells, ER+ breast cancer cells known to express SCGB2A2, were grown and treated with estradiol-17β 10^{-8} *M* in charcoal-stripped medium or with the antiestrogen ICI 182,780 (10^{-6} *M*) in regular medium for 6, 24, or 48 h, as previously described *(22)*. Total RNA was extracted from frozen tissue sections or cell lines using Tri-reagent (MRCI, Cincinnati, OH).

RT-PCR Analysis

One microgram of total RNA was reverse transcribed in a final volume of 20 μL and 1 μL of the reaction mixture subsequently amplified by PCR as previously described *(23, 24)*. Primers used corresponded to SCGB2A2 (sense 5'-CCGACAGCAGCAGCCTCAC-3', located in SCGB2A2 sequence between bases 41 and 59, and antisense 5'-TCCG TAGTTGGTTTCTCAC-3', located between bases 401 and 383) *(2)*; to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (sense 5'-ACCCACTCCTCCACCT TTG-3' and antisense 5'-CTCTTGTGCTCTTGCTGGG-3'); and to psoriasin *(24)* gene (sense 5'-AAGAAAGATGA GCAACAC-3' and antisense 5'-CCAGCAAGGACAGA AACT-3'). To amplify cDNA corresponding to SCGB2A2, GAPDH, and psoriasin, 30 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) of PCR were used. Ten microliters of PCR products were loaded on prestained (15 μg/mL ethidium bromide) 2% agarose gels. Identity of fragments corresponding to SCGB2A2, GAPDH, and psoriasin had previously been confirmed by sequencing.

Three independent PCRs were performed using SCGB2A2, psoriasin, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/ChemiDoc System (Biorad), were quantified by densitometry using the Quantity One software (Version 4.2, Biorad). SCGB2A2 and psoriasin expression were expressed relative to GAPDH expression as previously described *(25)*. Briefly, three independent PCRs were performed using each set of primers. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumor measured in each set of PCR experiments (always the same tumor) and all signals were expressed relative to this signal. Levels of SCGB2A2 were then expressed relative to the GAPDH signal corresponding to each individual tumor sample. Correlation between normalized SCGB2A2 expression and tumor characteristics was tested by calculation of the Spearman coefficient, *r*. Comparison between tumor subgroups was performed using the Mann–Whitney rank sum test, two-sided.

Immunohistochemical Analysis of SCGB2A2 Expression

Detection of SCGB2A2 protein was performed using an antibody previously characterized and kindly provided by Dr. Timothy Fleming *(1,2,5,18)*. Paraffin-embedded breast tissue sections were processed using the automated Discovery Staining Module, Ventana System (Tucson, Arizona) and the Research IHC DAB paraffin protocol according to the manufacturer's instructions. All steps were performed automatically: briefly, following deparaffination of tissue

sections, slides were incubated 60 min at 42°C in the presence of rabbit anti-SCGB2A2 antibody (1/1000 final concentration), washed, incubated with biotinylated secondary anti-rabbit antibody (14 minutes 42°C), washed, incubated 8 min with avidin–HRPO complex subsequently detected with DAB-H_2O_2 solution. Counterstaining was also performed automatically by the Ventana apparatus (hematoxylin/bluing reagent).

Levels of mammaglobin expression were assessed by bright-field microscopic examination at low-power magnification and using a previously described semiquantitative approach *(25)*. Scores were obtained by estimating average signal intensity (on a scale of 0 to 3) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one-tenth; 0.5, less than one-half; 1.0 greater than one-half). The intensity and proportion scores were then multiplied to give an overall score. Cases with a score lower than or equal to 1 were considered negative or weakly positive, whereas tumors with scores higher than 1.0 were classified as positive for SCGB2A2 expression. Statistical comparisons between tumor subgroups have been performed using the chi-square test.

Acknowledgments

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APPENDIX 2: Cancer Res. 2002. 62: 2736-2740

[CANCER RESEARCH 62, 2736–2740, May 15, 2002]

Advances in Brief

Identification of a Novel Breast- and Salivary Gland-specific, Mucin-like Gene Strongly Expressed in Normal and Tumor Human Mammary Epithelium[1]

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Abstract

Expression profiling using the public expressed sequence tag (EST) and serial analysis of gene expression (SAGE) databases resulted in the identification of a putative breast-specific mRNA that we have termed small breast epithelial mucin (SBEM). Hybridization analysis performed on 43 normal human tissues revealed that the *SBEM* gene was only expressed in mammary and salivary glands. Further reverse-transcription PCR analyses confirmed SBEM expression in most of established human breast epithelial cell lines analyzed (7 of 8) but not in cell lines of non-breast origin (0 of 6). *SBEM* mRNA expression was detected in >90% of invasive ductal carcinomas and correlated with the expression of a previously characterized breast-specific gene, mammaglobin-1 ($n = 54$; Spearman $r = 0.34$, $P = 0.011$). Interestingly, a higher SBEM:mammaglobin-1 ratio was observed in primary tumors with axillary lymph node metastasis than in node-negative tumors ($n = 46$; Mann-Whitney, $P = 0.04$). In a subset of 20 primary breast tumors and their matched axillary lymph nodes, a high concordance (Fisher's exact test, $P < 0.001$) was seen between PCR detection of *SBEM* mRNA in lymph node tissue and their histopathological status, indicating that SBEM mRNA expression is conserved in nodal metastasis. The *SBEM* gene is predicted to code for a putative low molecular weight, secreted sialoglycoprotein, potentially useful for the diagnosis of metastatic breast cancer.

Introduction

Early detection remains a central goal in breast cancer treatment to enable intervention at a localized and potentially curable stage and to maximize the opportunity for breast conservation. The 5-year survival rate for women with breast cancer increases dramatically when it can be diagnosed at an early stage, from >95% in patients with a localized tumor to ~75% with regional disease and <25% in women with disseminated cancer (1). Nevertheless, only 60% of all breast cancers are diagnosed at a local stage, and any improvement in early detection would have a significant impact on reducing overall breast cancer mortality.

Improving the diagnosis and clinical management of breast cancer requires access to a wider range of biomarkers able to reflect the molecular phenotype of breast tissue. A special need exists to identify novel genes whose expression is restricted to the mammary epithelium, because these genes have the greatest potential to enhance detection of micrometastatic disease and the potential to report on proliferative changes in the breast, analogous to the ability of elevated serum prostate-specific antigen levels to indicate the presence of hyperplasia or cancer of the prostate gland (2).

The identification of new tissue-specific markers has benefited especially from expansion of public and private databases for ESTs[4] (3, 4) and by large-scale efforts to profile patterns of gene expression using techniques such as serial analysis of gene expression (5). Using sequence analysis software and web-based tools developed for molecular profiling, we have identified a novel putative breast-specific gene, belonging to a recently regrouped cluster (UniGene identifier Hs.348419),[5] which represents an attractive candidate for a breast tumor marker with obvious potential for cancer diagnostics.

Materials and Methods

Database and Sequence Analysis. The cDNA xProfiler tool[6] was used to search for novel breast-specific ESTs. Protein sequence analysis used the SignalP algorithm[7] to search for the presence of a signal sequence (6) and the NetOGlyc algorithm[8] to predict sites of potential glycosylation (7).

RNA Hybridization Analysis for Tissue Specificity. A ^{32}P-labeled SBEM probe, generated using the cloned SBEM PCR product (396 bp) and the RadPrime DNA labeling system (Life Technologies, Inc., Burlington, Ontario, Canada), was hybridized to a commercially available RNA Master Blot (Clontech, Palo Alto, CA), containing poly(A)+ RNA (100–500 ng) isolated from a variety of adult and fetal human tissues, according to the manufacturer's instructions.

Cell Culture and RNA Preparation. Cell lines were obtained from the American Type Culture Collection or other sources and were cultured as follows: DMEM with 10% fetal bovine serum (MCF7, MCF10AT1, MCF10AT3c, SK-UT-1B, and HepG2); DMEM with 10% calf serum (MDA MB-231, Hec 1A, and HeLa); DMEM:Ham's F12 (1:1) with 10% fetal bovine serum (ZR-75-1 and RL95-2); RPMI 1640 with 10% fetal bovine serum (T-47D and LNCaP); or MSU-1 medium (8) with 5% fetal bovine serum (M13SV-1). All media were supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), HEPES (pH 7.4; 5 mM), and glutamine (2 mM). MCF7, T-47D, and ZR-75-1 cells also received bovine insulin (10 μg/ml). Media and sera were obtained from Life Technologies, Inc. (Life Technologies, Inc., Grand Island, NY). RNA was extracted from cultured cells using guanidinium isothiocyanate, followed by centrifugation through a 5.7 M cesium chloride cushion as described (9). RNA from cultured primary HMECs obtained by reduction mammoplasty was a kind gift from P. Ervin (Biotherapies, Inc., Ann Arbor, MI).

Breast Tumors and Axillary Lymph Nodes. Fifty-four invasive ductal carcinomas were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Cases spanned many ER (0–298 fmol/mg protein) and PR (0–1199 fmol/mg protein) levels, as determined by ligand binding assay. Tumors also spanned many grades (Nottingham grade scores from 5 to 9). For

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[4] The abbreviations used are: EST, expressed sequence tag; SBEM, small breast epithelial mucin; HMEC, human mammary epithelial cell; ER, estrogen receptor-α; PR, progesterone receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; MUC1, mucin 1.

[5] Internet address: http://www.ncbi.nlm.nih.gov/UniGene/. UniGene is a system for automatically partitioning GenBank sequences, including ESTs, into a nonredundant set of gene-oriented clusters.

[6] Internet address: http://cgap.nci.nih.gov/CGAP/Tissues/xProfiler.

[7] Internet address: http://www.cbs.dtu.dk/services/SignalP/.

[8] Internet address: http://www.cbs.dtu.dk/services/NetOGlyc/.

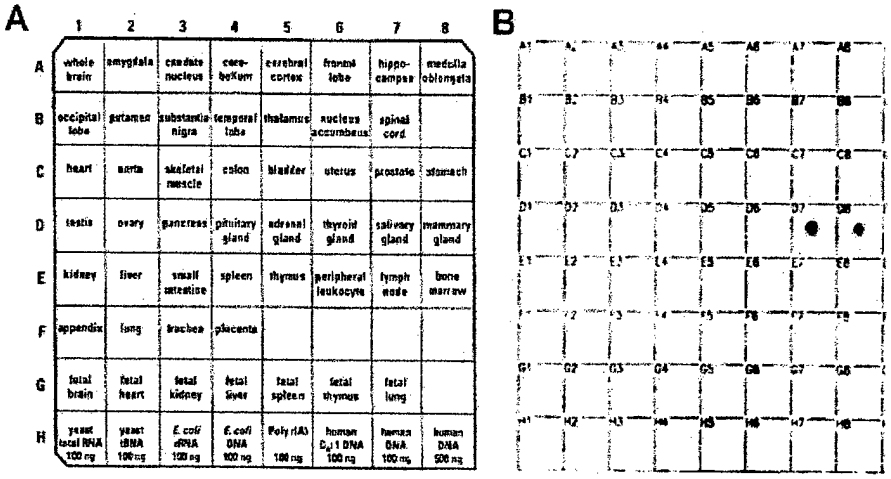

Fig. 1. Tissue expression of *SBEM* mRNA. An RNA Master Blot (Clontech Laboratories, Palo Alto, CA) containing poly(A)+ RNAs from different human tissues (*A*) was screened with ^{32}P-labeled SBEM probe as described in "Materials and Methods." The SBEM transcript (*B*) was expressed in the human salivary (*D7*) and mammary gland (*D8*).

46 tumors, pathological axillary lymph node status (presence or absence of metastasis) was known.

In a subset of cases (n = 20), frozen primary human breast tumor samples and their matched frozen lymph nodes containing (n = 14) or not (n = 6) histologically detectable metastatic cancer cells were available from the Manitoba Breast Tumor Bank. For the primary tumor samples, the ER levels, determined by ligand binding assays, ranged from 2.3 fmol/mg protein to 298 fmol/mg protein, whereas PR levels ranged from 10.1 fmol/mg protein to 112 fmol/mg protein.

RNA Analysis by RT-PCR. Total RNA was extracted from 20-μm frozen tissue sections (five sections/tumor) and reverse transcribed as described previously (10). The primers used for SBEM amplification consisted of SBEM-U (5′-CTTTGAAGCATTTTTGTCTGTG-3′; sense) and SBEM-L (5′-AAGGTAAGTAGTTGGATGAAAT-3′; antisense). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, aliquots of each reverse transcription mixture (2 μl for Fig. 2 and 0.8 μl for Fig. 3) were amplified in a final volume of 20 μl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 5 ng/μl of each SBEM primer, and 0.5 unit of Taq DNA polymerase. Each PCR consisted of 35 cycles (15 s at 94°C, 30 s at 58°C, and 60 s at 74°C).

Primers used for mammaglobin-1 were: Mam-1 (5′-CCGACAGCAGCAGCCTCAC-3′, sense strand) and Mam-2 (5′-TCCGTAGTTGGTTTCTCAC-3′, antisense strand). Primers for the ubiquitously expressed *GAPDH* gene were GAP-1 (5′-ACCCACTCCTCCACCTTTG-3′, sense strand) and GAP-2 (5′-CTCTTGTGCTCTTGCTGGG-3′, antisense strand). To amplify cDNA corresponding to mammaglobin-1 and GAPDH, 30 cycles of PCR were used (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C). All buffers were the same as for SBEM PCR, except that 2 mM $MgCl_2$ was used when amplifying mammaglobin-1 cDNA. PCR products were then separated on a 1.5% agarose gel. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml).

Quantification and Statistical Analysis. Three independent PCRs were performed for tumor specimens using SBEM, mammaglobin-1, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/Chemi-Doc System (Bio-Rad), were quantified by densitometry using the Quantity One software (version 4.2; Bio-Rad). SBEM and mammaglobin-1 expression was normalized to GAPDH expression as described previously (11). Correlation between SBEM expression and tumor characteristics or mammaglobin-1 expression was tested by calculation of the Spearman coefficient r. Differences between tumor subgroups were tested using the Mann-Whitney two-tailed test or Fisher's exact test.

***In Situ* Analysis of SBEM mRNA Expression.** *In situ* hybridization was performed on adjacent paraffin-embedded breast tumor tissue sections corresponding to a case shown to express high levels of *SBEM* mRNA by RT-PCR, using SBEM ^{32}P-labeled sense and antisense probes, as described previously (12).

Results

Identification of a Putative Novel Breast-specific Gene. The cDNA xProfiler tool (see "Materials and Methods") was used to identify tissue-restricted cDNAs with preferential representation in libraries prepared from normal breast tissue and breast tumors. This search identified a new cluster of ESTs now grouped under the UniGene identifier number Hs.348419. Of the 30 ESTs found in this cluster, 15 are ascribed to breast cDNA libraries, 9 were isolated from random activation of gene expression or pooled tissues, 5 were isolated from fetal sources (fetal heart and fetal skin), and 1 came from a head and neck tumor cDNA library. Alignment of these ESTs led to the construction of a 500-bp consensus cDNA sequence containing a 90-amino acid open reading frame in which the initiating methionine is framed by a nearly perfect consensus motif for translation initiation (5′-CCACCATGA-3′; Ref. 13). Further database analysis showed that this sequence, interrupted by three introns, is present on chromosome 12q13.2. Primers were designed to span the open reading frame, and we cloned a 396-bp fragment from both MCF-7 cells and breast tissue, which we called SBEM (GenBank accession number AF414087). The presence of a hydrophobic signal peptide (residues 1–19; Ref. 6) within the protein sequence (GenBank accession number AAL02119) suggests that SBEM is a secreted protein subject to proteolytic processing. The NetOGlyc glycosylation algorithm (7) further predicts this protein to be *O*-glycosylated on most of its 16 threonine residues. The SBEM protein contains three tandem copies of a neutral octapeptide core repeat (ThrThrAlaAlaXxxThrThrAla, where Xxx corresponds to Ala, Pro, or Ser). The NH_2 and COOH termini of the processed polypeptide are otherwise charged and fairly polar. These features suggest strong similarity to many sialomucins, although this protein lacks a transmembrane domain and is substantially shorter than most other known epithelial mucins (14, 15).

Expression of *SBEM* mRNA Is Restricted to the Mammary and Salivary Glands. Database searches suggested that SBEM expression was mainly restricted to breast tissue. To confirm this prediction, we performed hybridization analysis with an RNA MasterBlot containing highly purified polyadenylated RNA from 43 adult and 7 fetal human tissues arrayed on a nylon membrane. A *SBEM* cDNA probe hybridized exclusively to mRNA from the mammary and salivary glands (Fig. 1). Of note, no expression was observed in colon, lung, uterus, ovary, liver, pancreas, kidney, or prostate, all of which represent common primary tumor sites. Additionally, no hybridization to any of the fetal RNAs was observed.

***SBEM* mRNA Is Expressed in Breast Cancer Cell Lines but not in Cell Lines of Non-Breast Origin.** The profile of *SBEM* mRNA expression was further assessed using RT-PCR, followed by PCR amplification, in a panel of human breast and non-breast cell lines. A SBEM PCR product of the expected size (396 bp) was readily detected in MCF7 and ZR-75-1 breast tumor cells (data not shown). Lower but reproducible expression was also observed in primary HMECs and in several established breast epithelial cell lines including T-47D, M13SV-1 (8), MCF10AT1, and MCF10AT3c (16). MDA MB-231 breast tumor cells were negative for SBEM expression, as were six tumor cell lines of non-breast origin (uterus: RL95-2, SK-UT-1B, Hec 1A; cervix: HeLa; prostate: LNCaP; and liver: HepG2). As controls, we also examined the expression of a housekeeping gene (*GAPDH*) and mammaglobin-1, an established mammary-specific gene that is being independently investigated as a promising marker for breast tumor diagnosis and nodal metastasis (12, 17). Of the cell lines tested, only HMEC and ZR-75-1 cells expressed mammaglobin-1, consistent with published reports.

Analysis of *SBEM* mRNA in Human Breast Tumors. Northern blot analyses performed on a small series of 10 cases revealed that *SBEM* mRNA was 600 bp long and differentially expressed from one sample to another (data not shown). To determine whether *SBEM* mRNA was widely expressed in human breast tumor tissue, 54 human breast tumors, spanning many ER and PR levels as well as tumor grade and nodal status, were selected from the Manitoba Breast Tumor Bank. Total RNA was extracted from frozen tissue sections and reverse transcribed. PCR amplification of *GAPDH* (control), *mammaglobin-1,* and *SBEM* cDNA was then performed. A PCR product, 396-bp long was detected in all but three tumors (data not shown) when using SBEM-specific primers. After cloning and sequencing, this product was shown to correspond to *SBEM* cDNA. Quantification of the *SBEM* signal relative to the *GAPDH* signal was performed as described in "Materials and Methods." No correlation was found between SBEM expression and tumor characteristics such as ER ($n = 54$; Spearman $r = -0.01$, $P = 0.89$) and PR ($n = 54$; Spearman $r = -0.03$, $P = 0.77$) levels or tumor grade ($n = 44$; Spearman $r = -0.06$, $P = 0.68$). Interestingly, however, the *SBEM* signal correlated positively with *mammaglobin-1* expression ($n = 54$; Spearman $r = 0.340$, $P = 0.011$). Subgroup comparison of *SBEM* and *mammaglobin-1* expression confirmed our previous observation[9] that *mammaglobin-1* expression is higher in ER-positive and low-grade tumors (Table 1). Interestingly, although not statistically significant ($P = 0.09$), higher SBEM expression was found in lymph node-positive compared with node-negative tumors. Also of interest is the fact that the SBEM:mammaglobin-1 ratio is significantly ($n = 46$; Mann-Whitney, $P = 0.04$) higher in these lymph node-positive tumors.

[9] E. Leygue, L. C. Murphy, and P. H. Watson, unpublished results.

Table 1 *Median values (arbitrary units) of SBEM expression, mammaglobin-1 (Mam) expression, and SBEM:mammaglobin-1 ratio in different tumor subgroups*

Tumors	*n*	SBEM	*P*	Mam	*P*[a]	SBEM: Mam	*P*[a]
ER +	34	2.29	0.66	0.63	**0.04**	3.39	0.22
ER −	20	2.15		0.34		4.44	
PR +	34	2.30	0.63	0.59	0.27	3.56	0.50
PR −	20	1.97		0.38		3.58	
Node +	36	2.30	0.09	0.45	0.62	3.97	**0.04**
Node −	10	1.96		0.70		2.42	
Grades 5–7	24	2.05	0.84	0.59	**0.03**	3.33	0.10
Grades 8–9	20	2.24		0.34		4.02	

ER +, >3 fmol/mg of protein; ER −, ≤3 fmol/mg of protein; PR +, >10 fmol/mg of protein; PR −, ≤10 fmol/mg of protein, as determined by ligand binding assay. Node +, confirmed metastasis in axillary lymph nodes; Node −, absence of metastasis in analyzed axillary lymph nodes. Grade, Nottingham grading system. Subgroup comparison was performed using the Mann-Whitney two-tailed test.

[a] Bold face *P* values correspond to statistically significant differences between tumor subgroups ($P < 0.05$).

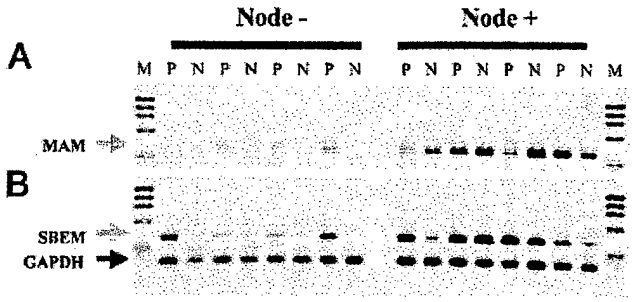

Fig. 2. RT-PCR analysis of *mammaglobin-1, SBEM,* and *GAPDH* mRNA expression in primary breast tumors (*P*) and their corresponding axillary lymph nodes (*N*), histologically shown to contain (*Node +*) or not to contain (*Node −*) metastases. *Mammaglobin-1* (*MAM*) PCR products were run separately (*A, dotted arrow*), whereas *SBEM* and *GAPDH* PCR products were mixed before separation on 2% agarose gels prestained with ethidium bromide (*B*). *Gray arrow,* product corresponding to SBEM; *black arrow,* product corresponding to *GAPDH. M,* molecular weight markers (Φ;x174 RF DNA/Hae III fragments; Life Technologies, Inc., Grand Island, NY). *N,* negative control, no cDNA added during the PCR reaction.

***SBEM* mRNA Expression in Primary Breast Tumors and Their Corresponding Axillary Nodes.** We next investigated the possibility that *SBEM* mRNA could be a tissue marker of axillary lymph node metastasis. Twenty independent cases were selected, including 14 tumors that were axillary lymph node positive and 6 that were node negative. Total RNA was extracted from frozen primary tumor sections and frozen node sections of corresponding axillary lymph nodes. The histological status of all tissues was confirmed in paraffin sections cut from adjacent mirror image paraffin tissue blocks that had been processed in parallel to the frozen blocks. These RNAs were reverse-transcribed and analyzed by RT-PCR using *SBEM*-specific primers. PCR was performed three times, giving the same result. A representative experiment is shown in Fig. 2. A signal corresponding to *SBEM* was detected in all lymph nodes containing metastatic cells by histopathological assessment (14 of 14 cases). In contrast, no signal was detectable in lymph nodes from cases without histologically detectable tumor cells (0 of 6 cases). RT-PCR detection of *SBEM* mRNA in axillary lymph nodes is therefore strongly associated (Fisher's exact test, $P < 0.001$) with the histopathological detection of lymph node metastases. The higher sensitivity afforded by RT-PCR detection therefore indicates that *SBEM,* perhaps together with *mammaglobin-1,* represents an excellent marker for the detection or confirmation of occult breast tumor metastasis, where histopathology may not be definitive.

***SBEM* mRNA Is Expressed in Mammary Epithelial Tumor Cells *in Vivo*.** To further establish whether *SBEM* was expressed by mammary epithelial cells *in vivo*, paraffin breast tumor tissue sections corresponding to a case shown to strongly express *SBEM* mRNA by RT-PCR were studied by *in situ* hybridization. No signal was detectable when using a sense probe (Fig. 3*A*). In contrast, a signal was observed in epithelial tumor cells when using an antisense probe (Fig. 3*B*). *SBEM* mRNA was not detected in stromal or inflammatory cells in any of the sections studied.

Discussion

This article reports the identification, cloning, and preliminary characterization of a cDNA encoding a novel mucin-like protein that displays an unusually narrow pattern of expression. Hybridization analysis revealed that *SBEM* mRNA was only detectable in two normal tissues, breast and salivary gland. Interestingly, the tissue-

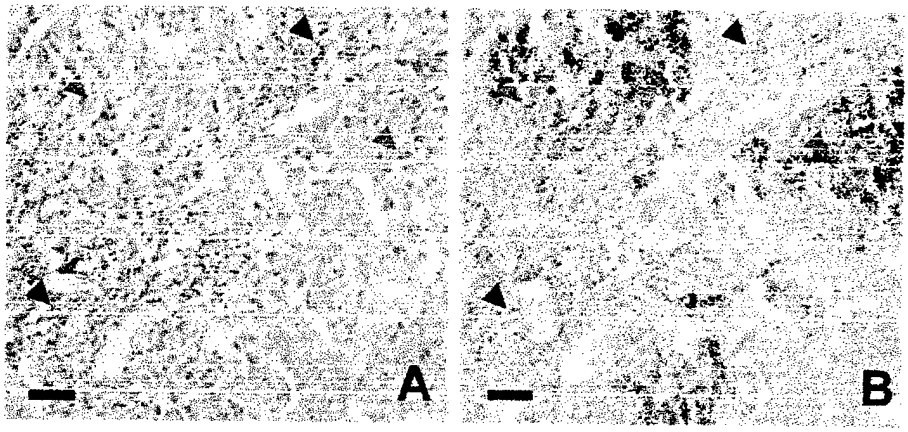

Fig. 3. Expression of *SBEM* mRNA in a primary breast tumor studied by *in situ* hybridization. These plates illustrate consecutive sections from a single breast tumor and show H&E-stained paraffin section treated with a sense probe (*A*) and *SBEM* mRNA expression in epithelial cells detected using an antisense probe (*B*). *Mauve* and *black arrowheads* show tumor epithelial cells and regions of stroma with inflammation, respectively. *Bar*, 30 μm.

specific expression that we observed experimentally directly reflects the distribution of ESTs within the Hs.348419 cluster. Indeed, as mentioned above, only two adult tissues (breast and head/neck tumors) have been shown to express SBEM-related ESTs. The fact that SBEM is also expressed in salivary tissue does not undermine the possible use of SBEM as a marker of breast cancer, because tumors of the salivary gland are less common and can readily be distinguished clinically.

Among the primary breast tumors examined in this study (representing mostly invasive ductal carcinoma), *SBEM* mRNA was observed by RT-PCR analysis in the majority (>90%) of cases. Despite a significant overall correlation between the expression of *SBEM* and *mammaglobin-1* mRNA, a significantly higher SBEM:mammaglobin-1 ratio was observed in primary tumors associated with positive axillary lymph nodes as compared with node-negative tumors. This was mostly attributable to a trend toward higher SBEM expression in node-positive tumors. Although further analysis of a larger number of tumors will be required to confirm these observations, this may suggest differences in the biology of these tumors and also a possible role of SBEM and mammaglobin-1 in the mechanisms involved in tumor metastasis. Our findings indicate, however, that SBEM expression is a common feature of breast cancer and can furthermore serve as a useful marker for breast nodal metastasis, both for detection of micrometastatic cells within lymph nodes as well as in the differential diagnosis of the primary origin of an unknown metastasis. This potential is enhanced by the conserved SBEM expression in high grade and ER/PR-negative tumors that are most likely to metastasize.

The potential diagnostic relevance of SBEM is also increased by its predicted biochemical structure. The *SBEM* cDNA sequence codes for a 90-amino acid polypeptide that contains a distinctive tandem repeat, rich in alanine and threonine residues, that represents a probable target for *O*-glycosylation. Consistent with such posttranslational modification is the presence of a well-defined signal peptide, leading us to predict that SBEM is likely to be processed at the apical surface of luminal epithelial cells and to be secreted into the alveolar or ductal lumen. Further study is needed to ascertain whether higher SBEM expression occurs in association with tumors.

Secreted (or transmembrane) proteins that contain internally repeated, densely glycosylated neutral core motifs such as this are characteristic of mucins, which are typically expressed by the surface epithelium of secretory mucosae and by exocrine glands (14, 15). The role of mucins is primarily one of hydrating and lubricating epithelial linings, although several mucins have been implicated in modulating both cell adhesion and growth factor signaling (18, 19). Furthermore, mucins have a well-established link to cancer, best illustrated by the product of the *MUC1* gene. *MUC1* is overexpressed in a variety of epithelial tumors including breast cancer and gives rise to several well-characterized tumor antigens including CA15.3 and CA27.29 (20, 21). Combined with the loss of cell polarity and changes in glycosylation patterns observed in transformed epithelial cells, overexpression of *MUC1* results in the appearance of mucin-derived tumor antigens in the sera of cancer patients that are not seen in normal controls (14, 22). We hypothesize that a similar situation may hold for SBEM expression in the human mammary epithelium and in human breast tumors. However, *MUC1* displays relatively broad expression among epithelial tissues including the colon, breast, pancreas, ovary, prostate, tracheobronchial tree, stomach, and uterus. For this reason, *MUC1*-derived tumor antigens have relatively poor specificity for individual tumor types, and their clinical utility is limited to monitoring the efficacy of cancer therapy and warning of tumor relapse or malignant spread (21–23).

Parallels between SBEM and known epithelial mucins such as *MUC1*, together with its more narrowly restricted pattern of expression, suggest that this novel gene represents an attractive candidate for a breast biomarker with potential for cancer diagnostics, as well as being a possible future target for the development of a breast tumor vaccine. Moreover, the absence of *SBEM* expression in normal lymph node tissue suggests that this gene could also be used to detect breast micrometastases in axillary lymph nodes.

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Appendix 3: Summary, CIHR 2003-2005

SUMMARY OF RESEARCH

In Canada alone, an estimated 21,000 women will be diagnosed in 2002 with breast cancer, and 5,400 women will be lost to this disease. There is an urgent need to identify novel genes whose expression is restricted to the mammary epithelium. Indeed these genes have the greatest potential (alone or combined with existing markers) to report on proliferative changes in the breast which may herald increased risk of breast cancer development, to enhance the detection of micrometastatic disease, and to be used for targeting specifically breast cancer cells.

We have identified a novel putative breast-specific gene, called hSBEM (human Small Breast Epithelial Mucin) which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics. We have indeed shown that hSBEM mRNA was expressed by breast tumor epithelial cells in 94% of primary breast tumors, independently of tumor characteristics such as steroid receptor levels or grade. We also demonstrated that hSBEM expression was conserved in breast tumor cells that metastasized in axillary lymph nodes. Interestingly, the protein encoded by this new gene shares structural similarities with MUC1, a heavily glycosylated protein over-expressed in epithelial tumors including breast and lung cancer. Change in MUC1 glycosylation patterns observed in transformed epithelial cells results in the appearance of mucin-derived tumor antigens in the sera of cancer patients. However, as MUC1 displays a broad expression among many normal epithelial tissues (including colon, breast, pancreas, ovary, prostate, and uterus), its clinical utility as a specific marker for breast cancer is limited. No data are available to date regarding the detection, the level of expression or the glycosylation states of the hSBEM protein during breast tumorigenesis.

HYPOTHESIS: hSBEM glycosylated protein, exists in both trans-membrane and C-terminally clipped soluble forms. As shown for MUC1, glycosylation patterns are changed during tumorigenesis. hSBEM protein is over-expressed during breast tumorigenesis and its increased concentration *in vivo* on the membrane of breast cancer cells and in their immediate vicinity can be used to pin-point in vivo the position of these cells.

GOAL: To establish whether the expression and/or the glycosylation pattern of hSBEM protein are modified during breast tumorigenesis and to develop an assay allowing the non invasive in vivo detection of breast cancer cells.

SPECIFIC AIMS

1 ***Investigation of hSBEM protein expression and glycosylation in breast cancer cell lines***
2 ***Investigation of hSBEM protein expression and glycosylation in normal and tumor breast tissues***
3 ***In vivo detection of hSBEM protein***

AIMS 1-2 hSBEM protein expression will be analyzed by Western blot and 2D gels following differential sugar removal in human breast cell lines and breast normal and tumor tissue. **Data will determine whether hSBEM gene expression is altered and whether changes in glycosylation profile occur during breast tumorigenesis.**

AIM 3 Iodinated or iron nanoparticles-coupled anti-SBEM antibodies will be injected in SBEM expressing tumor bearing mice. Preferential sites of antibody accumulation will then be located by counting and radiography or MRI. **Data will determine whether anti-hSBEM antibodies can be used to detect non invasively breast cancer cells in vivo in primary tumors and metastases.**

Appendix 4: Cancer Res. 2003. 63: 1954–1961

[CANCER RESEARCH 63, 1954–1961, April 15, 2003]

Psoriasin Interacts with Jab1 and Influences Breast Cancer Progression[1]

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ABSTRACT

Psoriasin (S100A7) is expressed at low levels in normal breast epithelial cells but is highly expressed in preinvasive ductal carcinoma *in situ*. Persistent psoriasin expression occurs in some invasive carcinomas and is associated with poor prognostic factors. Whereas there is evidence that secreted psoriasin can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions, an intracellular biological function is unknown. We have found that psoriasin physically interacts with Jab1 (c-jun activation-domain binding protein 1) in the yeast two-hybrid assay and confirmed this by coimmunoprecipitation assay in breast cancer cells. Psoriasin-transfected breast cancer cells showed increased nuclear Jab1 and demonstrated several features consistent with an alteration in Jab1 activity including an increase in activator protein-1 (AP-1) activity, increased expression of AP-1 and *HIF-1*-dependent genes, and reduced expression of the cell-cycle inhibitor $p27^{Kip1}$. Psoriasin overexpression was also associated with alteration of cellular functions that are associated with increased malignancy, including increased growth, decreased adhesion, and increased invasiveness *in vitro*, as well as increased tumorigenicity *in vivo* in nude mice. We conclude that intracellular psoriasin influences breast cancer progression and that this may occur through stimulation of Jab1 activity.

INTRODUCTION

We have identified psoriasin (S100A7) previously as a differentially expressed gene between DCIS[3] and invasive carcinoma (1). The expression of psoriasin is low in normal breast and benign pathologies (1), but psoriasin is among the most highly expressed genes in high grade DCIS (2, 3). Whereas expression is often reduced in invasive carcinoma, persistent high expression is associated with markers of poor prognosis (4). This profile of gene expression raises the possibility that psoriasin may be functionally involved in invasion and early tumor progression (5). Psoriasin is a small calcium-binding protein belonging to the S100 gene family (6, 7), among which several other members have been associated with breast tumor progression (8, 9). Most interest has been focused on *S100A4* (10). which was also initially identified as a differentially expressed gene between nonmetastatic and metastatic rodent mammary tumor cell lines (11). In later studies by several groups, *S100A4* has been shown to directly influence the invasive and metastatic phenotype in breast cancer cell lines (12–14) and tumors (15), and expression is also associated with poor prognostic factors and patient survival in human breast tumors (16).

Psoriasin was originally described as highly expressed within psoriatic skin lesions (17) and found to be a secreted protein (18), but has since been observed to be present in the cytoplasm and nucleus of both abnormally differentiated keratinocytes (19) and breast carcinoma cells (2, 4). Whereas there is evidence that secreted psoriasin can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions (18), a function for intracellular psoriasin also appears likely but has yet to be established.

We sought to identify proteins that might interact with psoriasin in breast epithelia by using the yeast two-hybrid system (20). Jab1 (21) was found to specifically interact with psoriasin in the yeast system, and this interaction was confirmed by biochemical assay in breast cancer cells. Jab1 is a component of a multimeric protein complex (22, 23), the CSN/COP9 signalosome, which is involved in signal transduction and protein degradation via the Ub-26S proteasome (24, 25). The effect of overexpression of psoriasin on Jab1 distribution and function in a breast cancer cell line was studied. Psoriasin overexpression resulted in redistribution of Jab1 to the nucleus and multiple functional changes that can be attributed to activation of Jab1, as well as enhanced tumorigenesis and metastasis in an *in vivo* assay. These data support our hypothesis that psoriasin enhances early tumor progression and the process of invasion in breast cancer cells in part by interacting with Jab1 and positively enhancing its activity.

MATERIALS AND METHODS

Yeast Two-Hybrid System. For yeast two-hybrid studies, the coding region of human psoriasin protein was fused in-frame with the GAL4 DNA-binding domain of the pGBT9 vector (Clontech). The resulting bait plasmid (pGBT9-psor) was used to screen a normal human mammary epithelium cDNA library (Clontech) by the yeast two-hybrid method as we have described previously (26). Clones were isolated that could grow on Trp^- Leu^- His^- medium, did not autoactivate the β-galactosidase reporter gene, and demonstrated specificity for their interaction with psoriasin. This was done by testing the interaction of psoriasin with specific "prey" constructs not identified in the screen. Jab1 was analyzed in a similar fashion. The NH_2-terminal "bait" psoriasin plasmid used to define the region of psoriasin involved in Jab1 binding encoded amino acids 1–52 (pGBT9-N-term-psor), and the COOH-terminal bait psoriasin plasmid encoded acids 43–101 (pGBT9-C-term-psor).

Cell Culture, Transfections, and Antibodies. The human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 were cultured in DMEM supplemented with 10% FBS under standard conditions (4). The former cell line is negative for psoriasin, whereas the latter expresses psoriasin mRNA and protein (confirmed by RT-PCR and Western blot; data not shown). The full psoriasin protein coding sequence was cloned into pcDNA3.1 (Invitrogen) and transfected into MDA-MB-231 cells using Superfect (Qiagen) followed by G418 selection. Resistant colonies were isolated and expanded. Psoriasin protein expression was determined by Western blot using a rabbit antipsoriasin antibody generated by our laboratory and directed against the epitope KQSHGAAPCSGGSQ corresponding to amino acids 88–101. The specificity of the antibody was established by comparison with a similar antibody generated previously against the same epitope (4), and by immunohistochemistry and Western blot, using transfected breast cancer cell lines and tumors as described previously (4). Three MDA-MB-231 clones were found to express psoriasin

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[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; RT-PCR, reverse transcription-PCR; AP-1, activator protein; VEGF, vascular endothelial growth factor; MMP3, matrix metalloproteinase 13/collagenase3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Jab1, c-jun activator binding protein-1; CAIX, carbonic anhydrase IX.

(designated as clone 231-LP1 exhibiting low psoriasin expression, and clones 231-HP1 and 231-HP2 both exhibiting similar high levels of psoriasin expression). Wild-type MDA-MB-231 and clone 231-neo (generated by transfection with the empty vector) do not express psoriasin. Jab1 and $p27^{Kip1}$ antibodies were obtained from Santa Cruz Biotechnology, Inc. Hypoxic stimulation of cells was performed in a Forma Scientific Model 1025 Anaerobic System containing an atmosphere of 0.7% O_2, 5% CO_2, and 5% H_2 at 37°C for 24 h as we have described previously (27).

Immunoprecipitation and Western Blot. Human breast cancer cell lines expressing psoriasin (231-HP2 and MDA-MB-468) were lysed on ice in 25 mM HEPES (pH 7.7), 0.4 M NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 1% Triton X-100, 0.5 mM DTT, and protease inhibitor mixture (Roche). Complexes were immunoprecipitated by Jab1 antibody/protein G-Sepharose (Pierce) at 4°C for 2 h. Binding and washes were performed in the same buffer, except the NaCl concentration was diluted 4-fold (28). Coimmunoprecipitated psoriasin protein was detected by immunoblotting using the psoriasin-specific antibody. Total protein lysates were extracted from the cell-line pellets in SDS-Isolation Buffer [50 mM Tris (pH 6.8), 20 mM EDTA, 5% SDS, 5 mM β-glycerophosphate, and a mixture of protease inhibitors (Roche)]. Protein concentrations were determined using the Micro-BCA protein assay kit (Pierce). Protein lysates were run on a 16.5% SDS-PAGE mini gel using Tricine SDS-PAGE to separate the proteins, and then transferred to 0.2 μm nitrocellulose (Bio-Rad). After blocking in 10% skimmed milk powder in Tris-buffered saline-0.05% Tween, blots were incubated with primary antibodies (~15 μg/ml in Tris-buffered saline-0.05% Tween) followed by incubation with appropriate secondary antibodies and visualization by incubation with Supersignal (Pierce) as per the manufacturer's instructions and exposure on X-ray films.

Reporter Gene Assay and Transcription of AP-1-dependent Genes. MDA-MB-231 parental cells and clones stably transfected with psoriasin were transfected with an AP-1-driven luciferase reporter gene (Stratagene) and a β-galactosidase expression vector, in triplicate experiments using Effectene (Qiagen). Luciferase was measured in cell lysates (Promega) 18 h after transfection and standardized to β-galactosidase activity (Promega). Total RNA from the MDA-MB-231 clones was isolated using TRIzol (Sigma) and reverse transcribed in duplicate from triplicate samples as described (4). Specific primers for VEGF, MMP13, and GAPDH were used for PCR as follows: VEGF-UPPER (sense) CGC AGA CGT GT AAA TGT TCC and VEGF-LOWER (antisense) AAG AAA AAT AAA ATG GCG AAT CC; MMP13-UPPER (sense) ATG CGG GGT TCC TGA T and MMP13-LOWER (antisense) CGC AGC AAC AAG AAA CAA; and GAPDH-UPPER (sense) ACC CAC TCC TCC ACC TTT G and GAPDH-LOWER (antisense) CTC TTG TGC TCT TGC TTG TTG G. Reactions were stopped during the log-linear stage of PCR amplification and samples electrophoresed through an agarose gel that was poststained with ethidium bromide for band visualization. Images were captured using an LCD camera and MCID software (Imaging Research, St. Catharine's, Ontario, Canada).

Immunohistochemistry. Cultured cells were grown on microscope slides for 24 h, and then fixed and processed as described previously (4). Immunohistochemical staining for psoriasin was performed essentially as described previously, using an automated tissue immunostainer (Ventana Medical Systems, Phoenix, AZ), and 3,3′-diaminobenzidine immunohistochemistry kit and bulk reagents supplied by manufacturer. Briefly, the staining protocol was set to "Extended Cell Conditioning" procedure, followed by 12 h incubation with primary antibody (concentration 1:3000) and 32-min incubation with secondary antibody. Positive staining was assessed by light microscopy.

Adhesion, Growth, and Invasion Assays. MDA-MB-231 clones were trypsinized from flasks that were 60–70% confluent. Cells (10,000) were plated in triplicate on three different days in 96-well plates having fibronectin, collagen I, or uncoated plastic surfaces (Becton Dickinson). After 1 h at 37°C, nonadherent cells were gently washed away with PBS. Adherent cells were stained with crystal violet, and their relative abundance determined by spectrophotometric absorbance. For growth assay, 1000 cells/well were plated in plastic 96-well plates in triplicate on three different days and allowed to grow for 18, 24, 48, and 72 h. Cells were stained with crystal violet and their relative abundance determined by spectrophotometric absorbance. Invasion assays were preformed in triplicate on a Matrigel-coated modified Boyden-invasion chamber (24-well plate inserts with 8-μm pores; Becton Dickinson). FBS DMEM (10%) was used as a chemoattractant in the lower chamber. Cells (350,000) were added to the upper chamber, and allowed 12 h to degrade the Matrigel and invade through the porous membrane. Cells that invaded and were adhering to the bottom of the membrane were stained with crystal violet. Invaded cells were visualized by light microscopy and numerated by counting the number of cells per high power field in five random fields.

***In Vivo* Mouse Studies.** Breast cancer cells (four experimental groups comprising MDA-MB-231 parental cells, 231-neo control, 231-LP1, and 231-HP1) were grown in culture and then suspended in 0.2 ml of PBS at a concentration of 5×10^5 cells before injection into mammary fat pads of female nude mice according to a protocol approved by the University of Manitoba Animal Care Committee. Each experimental group included 5 animals, and two injections were sited bilaterally in each animal to achieve a total of 10 possible tumor sites per group. Tumor diameters were measured by calipers at weekly intervals, and the tumor volume was calculated from the formula: volume = $4/3\ \pi\ (0.5 \times \text{smaller diameter}^2 \times 0.5 \times \text{larger diameter})$. The experiment was continued for up to 8 weeks at which time all of the animals were euthanized, and all of the injection sites, tumors, and multiple organ tissues (abdominal lymph nodes, lungs, liver, and spleen) were examined grossly for the presence of tumor. Representative tissue blocks from all of the primary injection sites and all of the organ sites suspicious for metastatic tumor were subsequently processed by 10% formalin fixation, paraffin embedding, and preparation of H&E-stained sections for light microscopic examination.

RESULTS

Identification and Confirmation of Psoriasin Interacting Proteins. We used full-length psoriasin fused to the GAL4 DNA-binding domain as bait in a yeast two-hybrid assay (20) and screened 1.74×10^7 clones from a normal human breast cDNA library. Among 4 true positive clones (26), 1 (Fig. 1*a*) contained almost the full protein sequence (amino acids 42–335) for Jab1. As shown in Fig. 1*a*, controls including unrelated bait (Rad18) and prey (Mad2) constructs, and empty bait and prey vectors did not show any activation of reporter genes. We noted that a Jab1-binding motif common to several Jab1 interacting proteins described recently is also contained within psoriasin (Fig. 1*b*), so we tested whether this region was necessary for the psoriasin-Jab1 interaction. As shown (Fig. 1*a*), only the COOH-terminal portion of psoriasin that contains this motif interacted with Jab1. To additionally confirm the psoriasin-Jab1 interaction in breast cancer cells, psoriasin was stably transfected into MDA-MB-231 cells, and coimmunoprecipitation experiments performed using Jab1 and psoriasin antibodies. Psoriasin-Jab1 protein complexes were detected in both psoriasin-transfected MDA-MB-231 cells (231-HP2) and the breast cell line MDA-MB-468 (which exhibits endogenous psoriasin expression) when Jab1 antibody was used for immunoprecipitation (Fig. 1*c*). However no psoriasin-containing complex was detected in control lanes in the absence of Jab1 antibody or protein G beads. Psoriasin-specific antibody immunoprecipitated psoriasin from cell lysates but was unable to coimmunoprecipitate Jab1 (data not shown), presumably because of the proximity and partial overlap of the epitope recognized by the antibody (amino acids 88–101 of psoriasin) and the proposed Jab1-binding domain (amino acids 57–89 of psoriasin).

Psoriasin and Jab1 Cellular Localization. We localized psoriasin and Jab1 in the MDA-MB-231 clones by immunohistochemistry. Jab1, like psoriasin, has been found previously to be both nuclear and cytoplasmic in cell types other than breast. In MDA-MB-231 cells and all 4 of the transfected cell lines (231-neo, 231-LP1, 231-HP1, and 231-HP2) Jab1 is expressed at comparable levels in the cytoplasmic compartment (Fig. 2, *right panel*). However, in all three of the psoriasin-expressing clones, 231-LP1, 231-HP1, and 231-HP2 (Fig. 2, *left panels*), there is a relative increase in Jab1 within the nucleus. However, the total amount of Jab1 protein as detected by Western blot is similar in all of the cell clones and does not change in the presence of psoriasin (Fig. 3*a*). Psoriasin can also be detected by immunoprecipitation of medium conditioned by 231-HP2 and MDA-MB-468

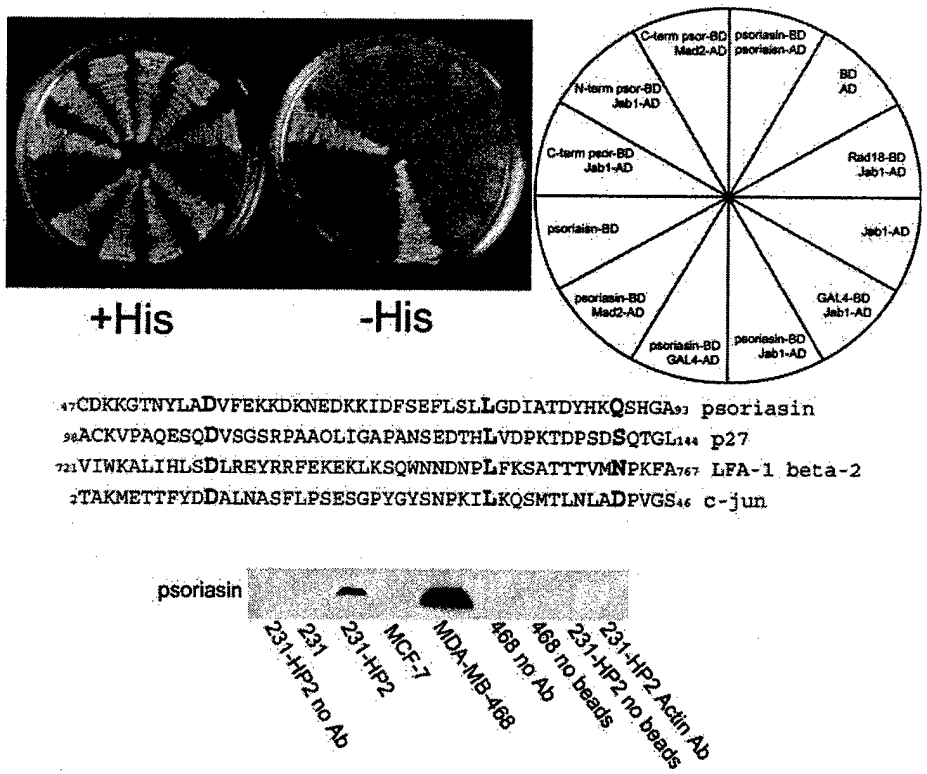


Fig. 1. Psoriasin specifically interacts with Jab1 in yeast two-hybrid assay and breast cancer cells. *a*, the psoriasin-Jab1 interaction was confirmed in yeast, by testing psoriasin fused to the GAL4-BD (binding domain) with selected proteins fused to the GAL4-AD (activating domain). Yeast plates (+ and − Histidine) are shown on the *left*, and the plating schema for each sector is shown on the *right*. Activation of reporter genes and colony growth is only present when the full-length psoriasin or the COOH-terminal half of psoriasin is combined with Jab1. *b*, the COOH-terminal half of psoriasin contains sequences similar to a Jab1-binding domain identified previously in $p27^{Kip1}$, LFA-1, and c-jun. *c*, psoriasin can be coimmunoprecipitated with Jab1 complexes from breast cancer cell lysates. Western blot using antipsoriasin antibody to detect psoriasin, which coimmunoprecipitated with Jab1, from a panel of breast cell lines (see "Materials and Methods").

cells (data not shown), suggesting that psoriasin is also secreted by breast cancer cells in culture.

Psoriasin Overexpression Influences Several Jab1-related Functions. Jab1 influences a number of cellular proteins. Among these, Jab1 effects the level of the negative cell cycle-regulating protein $p27^{Kip1}$ by promoting the export of $p27^{Kip1}$ from the nucleus to the cytoplasm and the subsequent degradation by the Ub-28S proteasome (29). Therefore, we first examined $p27^{Kip1}$ expression in our MDA-MB-231 clones and found that psoriasin-overexpressing clones showed a consistent reduction in levels of $p27^{Kip1}$ relative to wild-type and control cells (Fig. 3*a*).

To determine whether psoriasin influences other Jab1 functions in breast cancer cells we examined AP-1-dependent transcription in the MDA-MB-231 clones using an AP-1-driven luciferase reporter (Fig. 3*b*). AP-1 activity was increased in all 3 of the psoriasin-transfected clones in close proportion to the level of psoriasin expression (Fig. 3*a*). In the high psoriasin-expressing clones (231-HP1 and 231-HP2) there was a 6.5-fold increase in luciferase activity ($P < 0.0001$). These psoriasin-expressing cells showed no difference in total Jab1 levels assessed by Western blot, compared with non psoriasin-expressing controls (Fig. 3*a*). However, the effect on AP-1 activity is consistent with the redistribution and relative increase in nuclear Jab1 protein detected by immunohistochemistry (Fig. 2) and the findings of others (21). Expression of endogenous AP-1-dependent genes was next examined by RT-PCR (Fig. 3*c*). Psoriasin expression is also associated with an increase in mRNA levels of the endogenous AP-1-regulated genes VEGF (30) and MMP13 (31), and this increase is proportional to the levels of psoriasin in the MDA-MB-231 control and transfected cells.

Jab1 also interacts with HIF-1 (32) and enhances its activity. Expression of HIF-1 and the HIF-1-regulated gene *CAIX* (27) was examined by Western blot. Under hypoxic conditions (0.7% O_2), psoriasin-expressing clones showed a marked and higher induction of HIF-1 compared with control cells (Fig. 3*d, top panel*) and a parallel increase in CAIX protein (Fig. 3*d, middle panel*). However, it was noted that CAIX expression was also increased in psoriasin expressing 231-HP1 and 231-HP2 cells under normoxic conditions. The latter observation is consistent with the recent finding that CAIX can also be regulated by AP-1 (33) and indicates that a component of the CAIX induction seen under hypoxic conditions might be attributable to AP-1, given the involvement of AP-1 as well as HIF-1 in the cellular hypoxic response (34, 35).

Psoriasin Overexpression Influences Breast Tumor Progression *in Vitro*. We next looked for a relationship between psoriasin expression and biological end points relevant to tumor progression in breast cancer cells. The effect of psoriasin on growth of MDA-MB-231 cells was examined and found to be associated with a modest but significant increase in growth rate (Fig. 4*a*) of up to 1.3 fold ($P = 0.0009$). The influence of psoriasin on cellular adhesion, an important parameter of invasion, was measured in an *in vitro* assay. We observed a consistent reduction in cell-substrate adhesion (Fig. 4*b*) in psoriasin-expressing clones plated on plastic (0.42-fold reduction; $P < 0.0001$), collagen I (0.20-fold reduction; $P < 0.0001$), and fibronectin (0.18-fold reduction; $P < 0.0001$). The influence of psoriasin on invasion was then assessed in a modified Boyden chamber assay. There was a 1.4-fold increase in invasiveness in the high psoriasin-expressing clones ($P < 0.0001$) after 12 h (Fig. 4*c*), at which time there was no significant difference in growth (data not shown).

Psoriasin Overexpression Influences Breast Tumor Progression *in Vivo*. To determine whether psoriasin expression can also influence invasion and metastasis *in vivo*, psoriasin-overexpressing cells (231-LP1 and 231-HP1) and control cells (parental 231 and 231-neo) were injected into the mammary fat pad of nude mice, and the generation of tumors and metastasis was assessed (Fig. 5). Control cell lines (231 and 231-neo) generated tumors in 2 of 10 and 3 of 10 sites, respectively, after 8 weeks. These tumors were first noted between 2 and 3 weeks after injection, and increased slowly in size (Fig. 5*a*). Both psoriasin-expressing cell lines (LP1 and HP1) generated grossly detectable tumors in 7 of 10 and 6 of 10 sites. These tumors were also first noted between 2 and 4 weeks after injection but

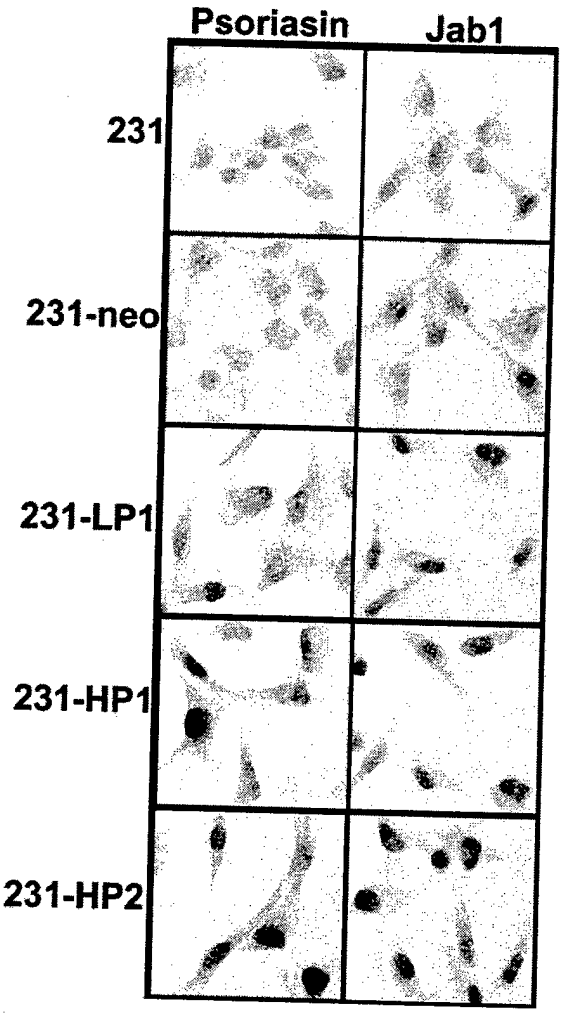


Fig. 2. Psoriasin expression is associated with redistribution of Jab1 to the nucleus. Psoriasin and Jab1 were detected in cells by immunohistochemistry, and representative fields of each cell line are shown. *Left panels* show lack of psoriasin expression in control MDA-MB-231 cells and 231-neo cells, and the expression in the psoriasin transfected clones (231-LP1, 231-HP1, and 231-HP2). *Right panels* show comparable cytoplasmic Jab1 expression in each of the corresponding cell lines, but with enhanced nuclear Jab1 in all three psoriasin overexpressing clones.

increased rapidly in size (Fig. 5, *b* and *c*). By week 8 there was no difference in incidence or mean tumor size between parental 231 cells and 231-neo controls, or between the two psoriasin-expressing clones (Fig. 5*e*). However, both psoriasin-expressing clones were significantly different from both parental and neo-transfected control cells ($P = 0.017$ and $P = 0.024$, Mann Whitney; Fig. 5*f*). Overall mean^{SD} tumor sizes (mm^3) for each experimental group were; MDA-231 = 21^{11}, 231-neo = 54^{8}, LP1 = 336^{223}, and HP1 = 370^{270}. When control groups and psoriasin transfectant groups were combined, the mean^{SD} tumor sizes (mm^3) were also significantly different: MDA-213 + 231-neo = 40^{20} and LP1 + HP1 = 352^{236} combined ($P = 0.0016$, Mann Whitney test). Microscopic examination of primary injection sites identified one additional microscopic tumor in the LP1 cell line group. The primary tumors derived from both control and psoriasin-expressing cells showed similar histological appearances. Expression of psoriasin was confirmed in representative tumors derived from psoriasin-transfected cell clones by immunohistochemistry (data not shown) and by Western blot (Fig. 5*d*). Psoriasin expression was only detected in tumors from psoriasin-transfected cells (although only a very weak signal was detected in the LP1 cell line). p27 expression was reduced in both psoriasin-transfected cell clone tumors. Grossly evident metastasis was identified and confirmed by microscopy in abdominal lymph nodes distant from the primary injection sites in 2 of 10 mice injected with psoriasin-overexpressing cells (both in the HP1 cell line group) compared with 0 of 10 mice in the control experimental groups.

DISCUSSION

The transition from normal epithelium through DCIS to invasive breast cancer is likely to involve many complex processes that are influenced by dynamic changes in gene expression (36). Perhaps the most critical of these processes is the acquisition of the invasive phenotype (37) that occurs with the transition from DCIS to invasive disease, because this event transforms an otherwise local disease into one that is capable of distant spread to threaten the host. It is likely that some of those genes that show alterations in expression between preinvasive and invasive components of breast tissues may be relevant to the process of invasion and offer markers of risk of early tumor progression (36). In this study we demonstrate that the psoriasin gene,

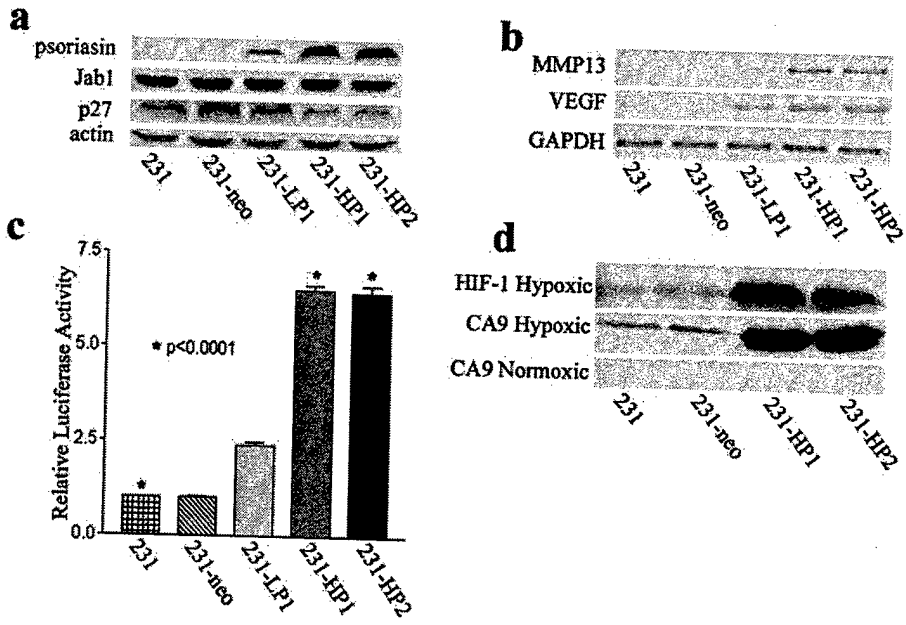
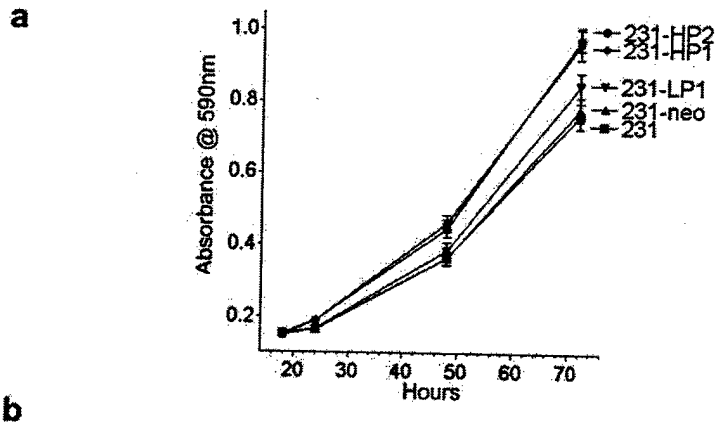


Fig. 3. The biological effects of psoriasin are mediated through Jab1. *a*, Western blot showing absent psoriasin expression in MDA-MB-231 and control 231-neo cells, and the relative levels of psoriasin expression in 3 transfected clones (231-LP1, 231-HP1, and 231-HP2). The total amount of cellular p27^{Kip1} is reduced in psoriasin-expressing clones, whereas the total amount of Jab1 does not change. Actin is shown as a loading control. *b*, psoriasin is associated with an increase in AP-1 activity, as tested by transfection of an AP-1-driven luciferase reporter plasmid. The relative increase in luciferase activity is proportional to the level of psoriasin in the MDA-MB-231 control and transfected cells. *c*, RT-PCR assay to show that psoriasin is also associated with an increase in mRNA levels of the endogenous AP-1-regulated genes VEGF and MMP13 that is also proportional to the levels of psoriasin in the MDA-MB-231 control and transfected cells. *d*, Western blot to shown that psoriasin expression is associated with up-regulation of HIF-1 and induction of the HIF-1-regulated gene, CAIX. Under normoxic conditions there is an increase in CAIX expression in psoriasin-expressing clones. Under hypoxic conditions there is a marked increase in induction of both HIF-1 and CAIX in the psoriasin-expressing clones; *bars*, ±SD.



b

Table 1- The Influence of Psoriasin on Cellular Adhesion to Selected Physical Surfaces

	Plastic	p-value	Collagen I	p-value	Fibronectin	p-value
231	1.000	-	1.000	-	1.000	-
231-neo	1.015 +/- 0.101	ns	1.008 +/- 0.065	ns	1.005 +/- 0.058	ns
231-LP1	0.752 +/- 0.089	p<0.0001	0.935 +/- 0.077	p=0.0027	0.941 +/- 0.067	p=0.0017
231-HP1	0.574 +/- 0.074	p<0.0001	0.802 +/- 0.076	p<0.0001	0.826 +/- 0.066	p<0.0001
231-HP2	0.591 +/- 0.080	p<0.0001	0.792 +/- 0.076	p<0.0001	0.819 +/- 0.065	p<0.0001

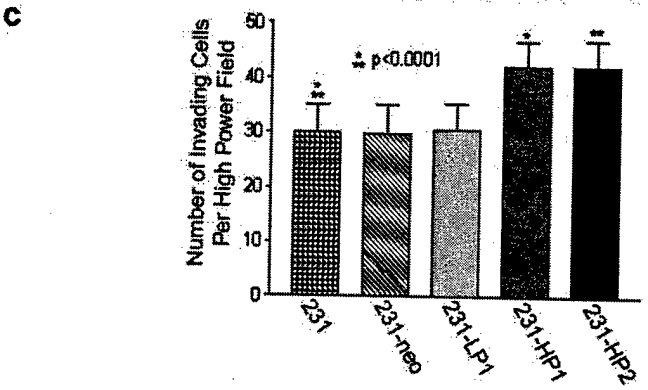


Fig. 4. Psoriasin expression alters cellular features associated with increasing malignancy in breast cancer. *a*, graph to show the growth rate of control cells (MDA-MB-231 and 231-neo) and relative increased growth of psoriasin-expressing clones (231-LP1, 231-HP1, and 231-HP2). Lines and data points represent the means of three independent experiments, with 12 data points at each time point within each experiment. *b*, cellular adhesion to plastic-, collagen I-, and fibronectin-coated surfaces is decreased significantly by psoriasin expression. Numbers represent relative adhesion to MDA-MB-231 cells on each substrate, and data represents the means of three independent experiments, with 12 data points for each cell line on each substrate. *c*, graph to show increased invasiveness in psoriasin-overexpressing cells in the *in vitro* Boyden chamber assay, which parallels the relative decrease in adhesion. *Columns* and *bars* represent means and SDs of three independent experiments where each cell line was assayed in triplicate.

which is highly expressed in DCIS and associated with poor prognostic factors when expressed in invasive disease, can enhance growth, adhesion, and invasiveness of a breast cell line in *in vitro* assays and tumorigenicity in nude mice *in vivo*. Furthermore, we describe a potential mechanism for these effects through a direct interaction between psoriasin and the multifunctional intracellular protein Jab1 (21).

Jab1 was originally identified in mammalian cells as a factor influencing c-jun transcription of AP-1-regulated genes (21). It soon became clear that Jab1 was also a component (CSN5) of a multimeric protein complex (22, 23). The CSN/COP9 signalosome had been studied previously in other systems and shown to be involved in protein degradation via the Ub-26S proteasome (24, 25). Jab1 has since been shown to be involved in a diversity of interactions with components of cell signaling pathways in *in vitro*, yeast, and human cell line model systems. These interactions appear to result in either translocation of Jab1 from cytoplasm to nucleus (integrin LFA-1 [38], erbB-2 [39] signaling), enhanced activity of transcription factors (including c-jun/AP-1 [21], HIF-1 [32], steroid receptors and cofactors [40, 41]) or the promotion of degradation of the interacting protein (including Smad4 [42], p53 [43], HIF-1 [32], MIF1 [28], and $p27^{Kip1}$ [29, 43]), often but not always associated with translocation from nucleus to cytoplasm. However, the physiological relevance of some of these interactions, and specifically in the context of breast epithelial cells, is mostly unknown.

In ovarian tumors, increased nuclear Jab1 is associated with progression and poor outcome (44), and altered Jab1 has also been implicated in renal cancer (45). A direct role for Jab1 in breast cancer has not been identified previously; however, several proteins including p53 and erbB-2, which are known to interact with or to influence Jab1, are altered at an early stage within high-risk DCIS (46–49) and may exert some of their effects through Jab1. The interaction between psoriasin and Jab1 also has the potential to directly facilitate several aspects of early tumor progression. We have shown here that overexpression of psoriasin is associated with translocation of Jab1 to the nucleus, alterations in expression of several Jab1 "downstream" genes, and increased proliferation, altered response to hypoxia, and promotion of invasion. Increased proliferation may be specifically attributable to increased AP-1 activity and down-regulation of the cell cycle inhibitor $p27^{Kip1}$ in this model. Alteration of Jab1 might also lead to increased activation of estrogen receptor and progesterone receptor, and up-regulation of cyclin D1 and alteration of transforming growth factor β signaling in other cell models (39, 50, 51), but these aspects of Jab1 function remain to be examined in the context of breast cancer. Increased capacity to survive hypoxic stress may occur through augmented HIF-1 activity and hypoxic response. Increased invasiveness may result from activation of AP-1 and HIF-1-dependent genes (52, 53), such as matrix metalloproteinases and VEGF, which are already implicated as critical factors in breast tumor progression (37, 54).

The estrogen receptor-negative MDA-MB-231 breast cell line was selected to reflect the context of psoriasin expression that we and others have observed previously in breast tumors *in vivo* (2, 4). The modest although significant increase in proliferation and invasiveness seen in our *in vitro* assays may reflect the fact that this cell line is already a highly proliferative and invasive cell in *in vitro* assay. More

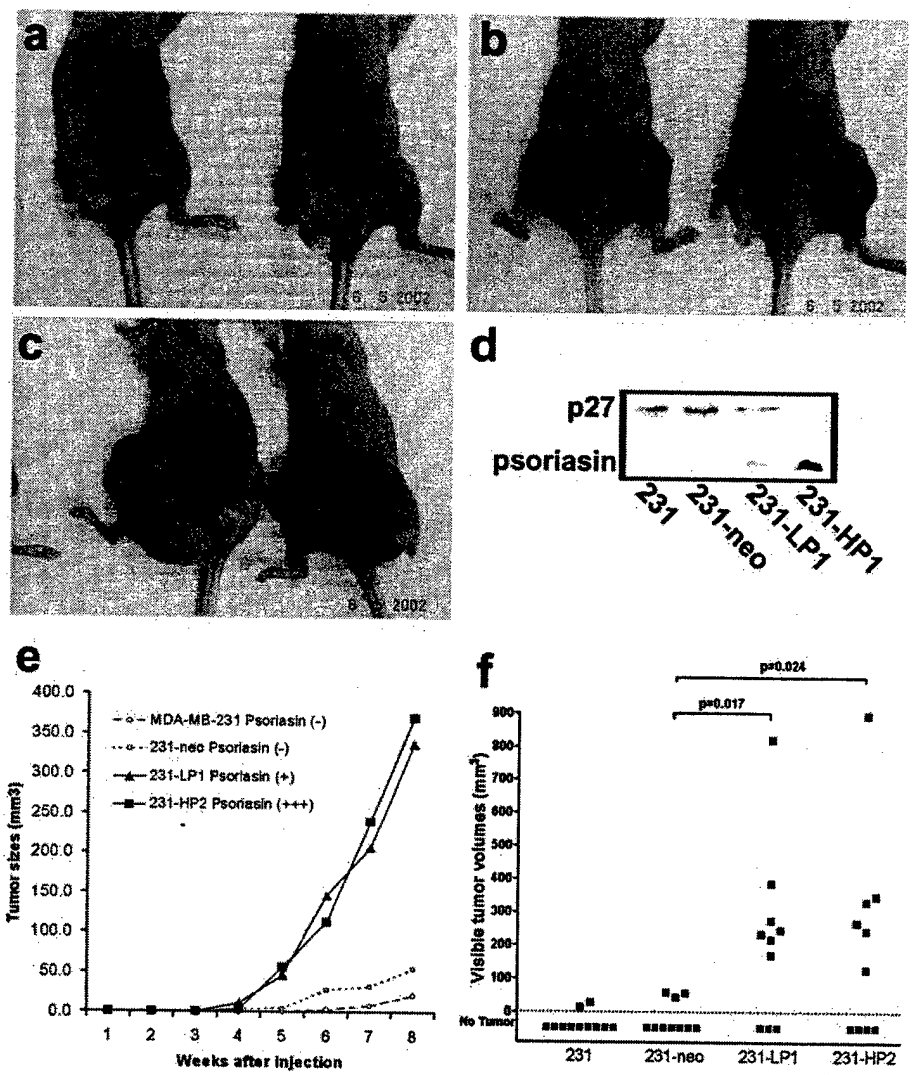


Fig. 5. Effect of psoriasin expression on tumor growth in nude mice. Groups of 5 mice for each cell line received an injection of 5×10^5 cells into the mammary fat pads. The *top panels* (*a–c*) show representative mice at 8 weeks from each treatment group that received (*a*) MDA-MB-231 cells, (*b*) 231-LP1 cells, or (*c*) 231-HP2 cells. *d*, expression of psoriasin and p27 protein determined by Western blot on extracts from representative tumors. *e*, relative growth curves for mice in each group. *Lines* and *data points* indicate the mean tumor volumes at each time point. *f*, distribution of tumor volumes at 8 weeks. Statistical significance was determined by Mann-Whitney test.

striking increases in growth and invasiveness were observed *in vivo* in the nude mice experiments, where metastasis was also associated with psoriasin-expressing tumors. This difference is consistent with the anticipated effects of enhanced metalloproteinase and VEGF expression on extracellular matrix and angiogenesis, spheres of influence that are not adequately replicated in *in vitro* assays, and has been observed by others studying the effects of overexpression of VEGF in breast cell lines (55). Nevertheless, additional detailed studies will be necessary to confirm the direct relationship and functional role of these specific factors in the enhanced growth and invasiveness seen in this model *in vivo*.

Alteration of Jab1 activity in tumors could be attributable in part to alterations in either the cytoplasmic-nuclear distribution (Refs. 38, 45, 56; as appears to be the case for the effect of psoriasin), the ratio of free Jab1:COP9-associated Jab1 (56), competition between different interacting proteins (42), or direct elevation of Jab1 expression and activation. The relevance of these potential mechanisms of action to breast cancer remains to be resolved, both for psoriasin and several other Jab1-interacting proteins. Nevertheless, it has been demonstrated that the many important activities of Jab1 can be influenced by competition between different interacting proteins (42). For example, p53 can compete with and down-regulate Jab1 activation of c-jun (57), and inhibition of Jab1 causes reciprocal up-regulation of p53 (42) and down-regulation of c-jun in HeLa cells (57). It is also interesting to note that the chemokine MIF can exert the opposite effect on Jab1 to psoriasin (28) with respect to modulation of AP-1 activity and $p27^{Kip1}$ expression. This raises the question of whether these different chemokine molecules might compete to modulate Jab1 activity.

Whereas our data support the involvement of Jab1 in mediating many of the biological actions of psoriasin, additional experiments will be needed to confirm that a direct interaction occurs between the putative Jab1 binding motif (29) on psoriasin and the Jab1 protein, and that direct alterations of Jab1 indeed exert effects on these specific target genes and pathways. It is also possible that some of the functions of psoriasin are mediated through other pathways (58). For example, it has been shown that other secreted S100 proteins (S100B and S100A12) can bind to and stimulate the receptor for advanced glycation end products, leading to activation of intracellular signaling pathways including up-regulation of ras, mitogen-activating protein kinase and nuclear factor $\kappa\beta$ in immune cells (59, 60). Expression of receptor for advanced glycation end products is also associated with invasion in gastric carcinoma (61) and is functionally involved in metastasis (62). Unlike some other S100s with chemokine activities such as S100A9 and S100A12, which are expressed by both epithelial and stromal inflammatory cells (63), expression of S100A7 (psoriasin) is restricted to epithelium, at least in skin and breast. However,

psoriasin is also secreted and could potentially interact with cell surface receptors on immune or epithelial cells.

In summary, we have shown that psoriasin can contribute to breast tumor progression and that its action may be mediated, at least in part, through Jab1. Although other important cellular proteins also influence and may compete for Jab1, psoriasin is one of the most abundant proteins in high-risk DCIS (2) and is likely to exert an important effect on Jab1 activity in breast tumor cells at an early stage of tumor progression. Thus, therapies aimed at modulating the effect of psoriasin may have important potential in the treatment of early breast cancer.

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Putative functional characteristics of human estrogen receptor-beta isoforms

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Abstract

Estrogen receptors (ERα and ERβ) are clearly multifaceted in terms of structure and function. Several relatively abundant ERβ isoforms have been identified, which can be differentially expressed in various tissues. In order to provide insight into the possible role of the ERβ family in breast tissue a study of the putative functions of the human (h) ERβ1, hERβ2 and hERβ5 isoforms was undertaken. Only hERβ1 was found to bind ligand, which induced conformational changes as determined by protease digestion assays. All ERβ isoforms could bind to and bend DNA although the relative efficiency with which they bound DNA differed with hERα>hERβ1>hERβ2>>hERβ5. All ERβ isoforms inhibited ERα transcriptional activity on an estrogen-response element (ERE)-reporter gene. The relative activities were hERβ1>hERβ2 >hERβ5; however, only hERβ1 had transcriptional activity of its own. Both LY117018–hERα and LY117018–hERβ1 complexes alone could activate transcription on a TGF-β3-CAT gene. Although hERβ2 and hERβ5 had no activity alone, they inhibited ERα but not hERβ1 transcriptional activity of transforming growth factor (TGF)-β3-CAT. In marked contrast to activity on an ERE-CAT reporter gene, hERβ1 did not modulate ERα transcriptional activity on a TGF-β3-CAT reporter gene. These data support promoter-specific differential activities of hERβ isoforms with respect to models of ERα regulated gene expression, and suggest that they may have a role in differentially modulating estrogen action.

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Introduction

The estrogen receptor family of steroid hormone receptors is clearly multifaceted (Hall *et al.* 2001) and more complex than originally thought. There are two genes which encode estrogen receptors (ER), ERα and ERβ. Both are ligand regulated transcription factors which classically modulate target gene transcription by binding as homo- and/or heterodimers to estrogen responsive sequences in target gene promoters (Cowley *et al.* 1997). These receptors likely have distinct roles in estrogen action, independent of each other when they are expressed separately (Couse & Korach 1999) but can also have direct interactions due to heterodimerization when the receptors are expressed together in the same target cell (Enmark *et al.* 1997). In addition, both ERs may encode variant isoforms generated by alternative splicing mechanisms (Lu *et al.* 1998, Moore *et al.* 1998). In particular there are data to support variant isoforms of ERβ at the protein level (Fuqua *et al.* 1999, Fujimura *et al.* 2001). Furthermore, we have shown in human breast tissues that variant forms of ERβ are more abundant than the wild-type at least at the RNA level (Leygue *et al.* 1999).

Human (h) ERβ2 (also called hERβcx (Ogawa *et al.* 1998*b*)) and ERβ5 variant mRNAs are missing the wild-type exon 8 sequences and contain extra sequences which are distinct from each other, followed by sequences that are then identical with each other (see Fig. 1). They are predicted to encode C-terminally truncated ERβ-like proteins identical to wild-type until amino acid residue 468 (by reference to the long form of hERβ1) (Ogawa *et al.* 1998*a*). After amino acid 468 hERβ2 is predicted to encode 28 novel amino acids, with the full-length protein having a predicted molecular mass of 55·5 kDa. In contrast, after amino acid 468 hERβ5 is predicted to encode only 5 novel

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amino acids with the full-length protein having a predicted molecular mass of 53 kDa.

Although total ERβ expression appears to decrease between normal breast and ER-positive breast tumors (Leygue *et al.* 1998*b*, Roger *et al.* 2001), the relative expression of the variant ERβ isoforms to the wild-type ERβ can also change during breast tumorigenesis, at least at the RNA level (Leygue *et al.* 1999). This suggests that the expression and/or the activity of the ERβ family of receptors changes during breast tumorigenesis and may have a role in this process as well as having a role in the altered estrogen action that occurs during breast tumorigenesis. In order to provide insight into the possible role of the ERβ family in breast tissue we have undertaken a study of the putative functions of the hERβ1, hERβ2 and hERβ5 isoforms.

Materials and methods

Materials

17β-Estradiol (E_2), 4-hydroxytamoxifen (4-OH-TAM) and CAPS (3-cyclohexylamino-1-propanesulfonic acid) were from Sigma Chemical Co. (St Louis, MO, USA). ICI 182,780 was a gift from Dr A E Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, UK). LY117018 was a gift from Eli Lilly Co. (Indianapolis, IN, USA). [^{3}H]17β-Estradiol, [^{14}C]chloramphenicol, and [^{35}S]-methionine were from New England Nuclear (Boston, MA, USA). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{35}S]-ATP were from ICN Pharmaceuticals (Irvine, CA, USA). All cell culture reagents were obtained from GIBCO/BRL (Burlington, Ontario, Canada).

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System; Promega, Madison, WI, USA). Reactions were performed according to the manufacturer's instructions.

Scatchard analysis

Human ERα (pcDNA3·1/wild-type human ERα from HEGO (Green *et al.* 1986)), human ERβ1 (pcDNA3·1 hERβ1, long form of 530 amino acids (Leygue *et al.* 1998*a*, Ogawa *et al.* 1998*a*)), human ERβ2 (pcDNA3·1 hERβ2, long form (Ogawa *et al.* 1998*b*)) and human ERβ5 (pcDNA3·1 hERβ5, long form) proteins were synthesized by *in vitro* transcription–translation as described above. Ligand binding studies were conducted as previously described (Lu *et al.* 2000). *In vitro*-generated receptor was diluted 10-fold in buffer (10 mM Tris-HCl, pH 7·5, 1·5 mM EDTA, 10 mg/ml BSA, 10% glycerol) and kept on ice until use. One hundred microliters of the diluted protein were used in each binding reaction that contained varying concentrations of [^{3}H]E_2 (0·01–100 nM), followed by overnight incubation at 4 °C. Non-specific binding was determined by parallel incubations containing a 200-fold excess of unlabeled E_2. Unbound steroid was removed by addition of 500 μl 0·5% charcoal–0·05% dextran in the above dilution buffer for 30 min at 4 °C followed by centrifugation at 10 000 × ***g*** for 10 min at 4 °C. Radioactivity was determined in an aliquot of the supernatant and in aliquots of total [^{3}H]E_2 solutions using a scintillation counter. The ratio of specifically bound/unbound steroid and the concentration of specifically bound steroid were used for Scatchard analysis, from which was determined the equilibrium dissociation constant, K_d.

Limited proteolytic digestion analysis

Conformational studies were performed as described previously (Beekman *et al.* 1993). *In vitro*-synthesized ERs were incubated with agonists (E_2, diethylstibesterol) and antagonists (4-OH-TAM, LY117018) overnight at 4 °C. The liganded receptors were then diluted 1:10 (v/v) in TE buffer, then 20 μl of this ER solution were treated with increased concentrations of trypsin (0·2 to 5 μg) for 20 min at room temperature and stopped by the addition of loading buffer. The samples were boiled and were analyzed directly by SDS-polyacrylamide gel electrophoresis (10% w/v). The gel was dried and the digested bands were visualized by autoradiography.

Electrophoretic mobility gel-shift assay (EMSA)

In vitro-synthesized human ERs were used for EMSA. Typically 1 μl programmed lysates containing equal amounts of each receptor as determined

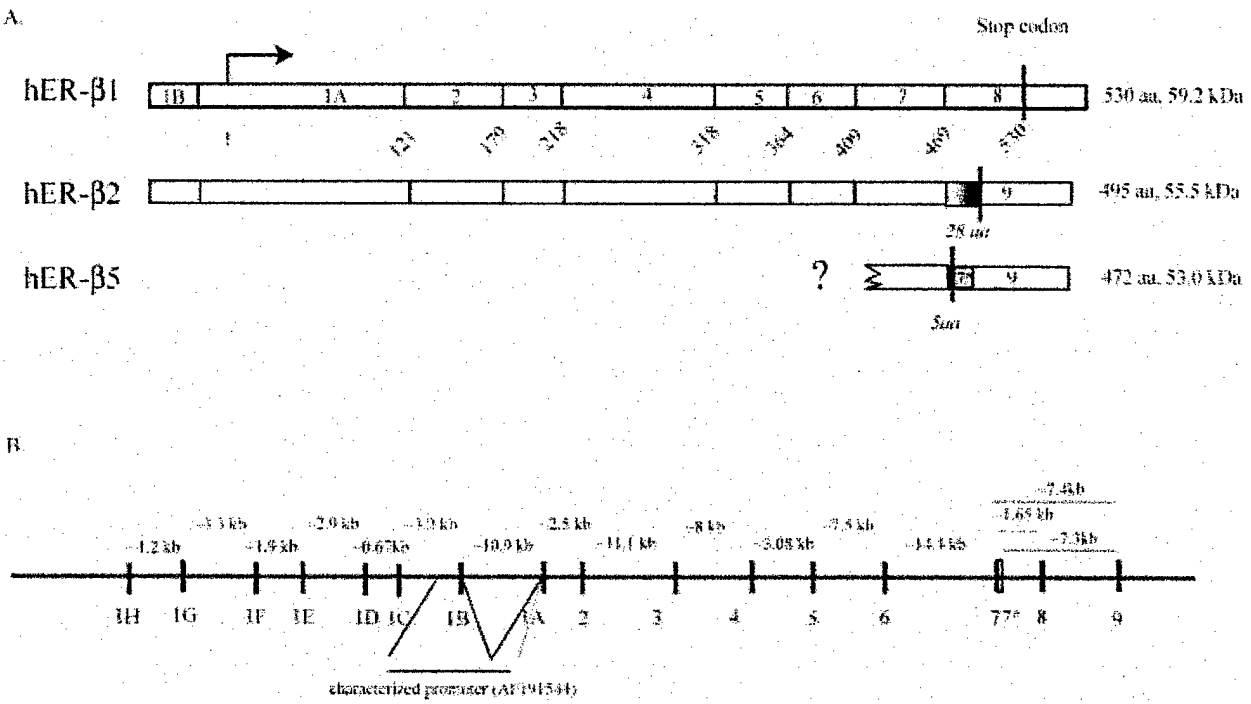


Figure 1 (A) cDNA structure of human ERβ1 (hER-β1), human ERβ2 (hER-β2) and human ERβ5 (hER-β5) and predicted proteins of the human ERβ isoform cDNAs. Genomic structure of the human estrogen receptor β locus on chromosome 14. Human ERβ cDNA, expressed sequence tags and published promoter sequences (accession numbers in the text) were aligned with sequences from two genomic clones of human chromosome 14 (AL162756/CNS01 RHJ and AF215937). Exons 1C to 1H are found only in one cDNA (accession number AB006589). aa, amino acids.

by polyacrylamide gel electrophoresis of ^{35}S-methionine-labeled protein generated in parallel *in vitro* transcription–translation assays, was assayed in EMSA. One microliter lysate was incubated in a final volume of 20 µl, and the reaction solution was 5 mM Tris-HCl, pH 7·5, 100 mM KCl, 2 mM dithiothreitol, 5% v/v glycerol and contained 2 µg polyd(I-C). The binding reaction was initiated by adding 1 µl (approx. 10 fmol) 5′-[^{32}P]-end-labeled, double stranded estrogen-response element (ERE) oligonucleotide (35 mer, 5′-AACTTTGATCAGG TCACTGTGACCTGACTTTGGAC-3′ containing the vitellogenin A2 ERE sequence), and the mix was incubated at 20 °C for 30 min. DNA-bound complexes were electrophoretically separated on a 4·5% loosely cross-linked acrylamide gel (1:29 bis:acrylamide) at 150 V for 90 min at 20 °C in 0·5 × TBE buffer. Gels were then vacuum dried and autoradiographed. To identify immunoreactive ER within retarded DNA-bound complexes, parallel incubations containing 1 µg ER antibody (usually 1 µl H222 for ERα, or 1 µl PAI-310 for ERβ1 and ERβ2) were run to determine the presence of super-shifted antibody-bound ER–ERE complexes (data not shown). Reticulocyte lysates containing *in vitro*-translated ER proteins were incubated with or without saturating concentrations of ligand (estrogen or antiestrogen) at 4 °C overnight to allow receptors to bind ligand, followed by EMSA.

DNA bending assay

The DNA bending vector ERE Bend I (kindly provided by Dr A Nardulli, University of Illinois, Urbana, IL, USA) (Nardulli & Shapiro 1992) was digested with EcoRI and EcoRV to produce a 430 bp DNA fragment with a single consensus ERE either at the end (EcoRI fragment) or in the middle (EcoRV fragment). The fragments were then gel purified, labeled by incubation with polynucleotide kinase in the presence of

[γ-^{32}P]ATP, and purified on a G50 Sephadex column. Gel mobility shift assays were carried out essentially as described above. Aliquots of the binding reactions were run on 8% non-denaturing acrylamide gels, dried and exposed to X-ray film. The degree of DNA bending was determined using the method of Thompson and Landy (1988).

Cells, cell culture and transient transfection

For transient transfection analysis, tagged ER expression vectors were generated. Human ERα, ERβ1, ERβ2 and ERβ5 were tagged at their N-terminus with a polyhistidine and an Xpress epitope tag using the pcDNA4/HisMax(A) plasmid (Invitrogen Canada Inc., Burlington, Ontario, Canada). Cos-1 and Cos-7 cells were obtained from the ATCC (Manassas, VA, USA). The cells were routinely cultured in DMEM containing 5% v/v fetal calf serum (FBS), 1% w/v glucose, glutamine and penicillin–streptomycin (5%CM). To obtain estrogen-depleted cells, the culture medium of stock cells was changed to phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS, 1% w/v glucose, glutamine and penicillin–streptomycin (5%CS) and replaced every 2 days. Six days later the medium was replaced by 10%CS until required for experiments. For transient transfection experiments, the cells were transfected using the Effectene transfection reagent according to the manufacturer's instruction (QIAGEN, Mississauga, Ontario, Canada). Briefly, the day before transfection, the estrogen-depleted cells were seeded in 6-well plates at $2{\cdot}5 \times 10^5$ cells per well in 2 ml 5%CS and left overnight. The plates were 70–80% confluent on the day of transfection. The transfection mixture was prepared according to the manufacturer's protocol, then fresh medium (5%CS) was added to the transfection mixture and 0·6 ml per well of the above mixture with either ERE-II-TCO-CAT (a gift from P Webb (Webb *et al.* 1995)) or transforming growth factor (TGF)-β3-CAT-reporter plasmid DNA (Yang *et al.* 1996) was added. ER expression plasmid (50–450 ng) or empty vector and 100 ng β-gal pCH110 plasmid DNA (Pharmacia Canada, Mississauga, Ontario, Canada) was added drop-wise into the medium (CS) and the plates were gently swirled to ensure uniform distribution of the DNA–Effectene complexes. Vehicle (ethanol), estradiol-17β or LY117018 was then added 20 to 30 min later. The cells were left for 48 h and then harvested. Cell extracts were prepared by freeze/thawing and were used to determine chloramphenicol acetyl transferase (CAT) and β-galactosidase activity as previously described (Dotzlaw *et al.* 1992).

Western blot analysis

For Western blot analysis, $2{\cdot}5 \times 10^5$ Cos-1 cells were set up in 6-well plates, then transiently transfected with plasmids and treated with estrogen or antiestrogen under the same conditions as for the CAT assay described above. Cells were harvested 48 h after transfection, washed once with Isoton II and then the washed cell pellets were resuspended in 200 μl Isoton II. Aliquots of cell suspension (150 μl) were extracted and used for Western blots and the remainder was used for determination of β-galactosidase activity. For Western blotting, the cells were pelleted and then extracted using 40 μl hot (95 °C) extraction buffer J with shaking for 20 min at 95 °C as previously described by Joel *et al.* (1998). The entire extract was subjected to 10% SDS-polyacrylamide gel electrophoresis as previously described (Adeyinka *et al.* 2002). The separated proteins were transferred to nitrocellulose membranes and processed as previously described (Adeyinka *et al.* 2002). Detection of the tagged estrogen receptor proteins was by incubation of blots with anti-Xpress antibody (1/5000 in TBST, Cat#R910-25, Invitrogen Canada Inc.) overnight at 4 °C, followed by washing and incubation with secondary antibody (horseradish peroxidase conjugated goat anti-mouse antibody, 1/5000 in TBST, Jackson Immuno Research Labs Inc., West Grove, PA, USA) at room temperature for 2 h. Visualization was carried out using the SuperSignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Statistical analysis

Differences in variance were tested using ANOVA, where appropriate. Differences between individual mean values were then determined using Student's *t*-tests. All tests were performed using GraphPad Prism statistical analysis software (GraphPad Software Inc., San Diego, CA, USA).

Results

Identification and organization of hERβ1, hERβ2 and hERβ5 cDNA sequences at the hERβ locus on chromosome 14

The estrogen receptor beta gene has been localized to human chromosome 14q22–24 and the genomic structure of 8 exons comprising hERβ1 has previously been published (Enmark *et al.* 1997). However, the previously described variant hERβ2 (also called hERβcx) and hERβ5 mRNA contain only sequences corresponding to exons 1 to 7 of hERβ1 and then they diverge (see Fig. 1A). They do not contain exon 8 sequences of hERβ1, but contain sequences termed exon 9, which are located downstream of exon 8 on chromosome 14 (Fig. 1B), identified using database sequences of chromosome 14 (accession numbers CNSO1 RHJ and AF215937) and the Human Genome Working Draft. It should be noted that neither of these genomic sequences contain an extra A 5′ of the start site of translation for hERβ, that would place another upstream ATG in frame with the known coding region and introduce 18 amino acids to the N-terminal of the known coding region, as recently described (Wilkinson *et al.* 2002). Interestingly, hERβ5 mRNA also contains sequences between exon 7 and part of exon 9 which are not present in either hERβ1 or hERβ2 mRNA. These hERβ5 mRNA specific sequences can be found immediately following exon 7 sequences in intron 7 of the human ERβ gene (Fig. 1B), suggesting that the normal splice donor site is not recognized and a cryptic splice donor site is present in intron 7. Furthermore the exon 9 sequences present in hERβ5 cDNA start 28 nucleotides downstream of those present in hERβ2, suggesting a cryptic splice acceptor site is present within exon 9. There are also multiple non-coding exons 5′ to exon 1 as previously identified (Enmark *et al.* 1997), since several hERβ cDNAs contain sequences in their 5′ UTR which are found further upstream of the previously described exon 1 on chromosome 14 (Fig. 1B; 1H–1C seen in AB006589, 1B seen in NM_001437, AX234658, AF05428, AF060555, AB006589 references). The sequences of a recently characterized promoter region of hERβ (Li *et al.* 2000) are found immediately upstream and overlapping with exon 1B. However, the presence of hERβ mRNAs whose 5′UTR contain exonic sequences found upstream of this documented promoter suggest that there are alternative promoters for the hERβ gene. This is similar to the hERα gene and suggests that regulation of expression of these genes is complex (Kos *et al.* 2001).

The predicted open reading frames for hERβ1, hERβ2 and hERβ5 are shown in Fig. 1A. hERβ2 contains amino acids 1–468 which are identical to hERβ1; the sequence then diverges containing another 28 novel amino acids encoded in the open reading frame. hERβ5 was isolated as a partial cDNA but is likely also to be identical to hERβ1 from amino acids 1–468 and then diverges containing another 5 novel amino acids. Both these variant hERβ proteins would be truncated at the C-terminus, disrupted in helix 11 and missing helix 12 and therefore unlikely to bind ligand or have AF2-mediated transcriptional activity. Lack of ligand binding has been confirmed using *in vitro* generation of these proteins as outlined below.

Ligand binding activity of hERβ1 and variant isoforms hERβ2 and hERβ5 proteins

Human ERβ1 has previously been shown (Enmark *et al.* 1997) to bind E_2 with high affinity and specificity, and our data confirm these findings. Figure 2A shows specific saturable binding of [^{3}H]E_2 to *in vitro* translated hERβ1 with a calculated K_d=0·11 nM. However, the open reading frames of hERβ2 and hERβ5 cDNA predict for C-terminally truncated proteins compared with hERβ1 and are predicted not to bind ligand. As shown in Fig. 2B and C no saturable binding of [^{3}H]E_2 to *in vitro*-translated hERβ2 or β5 was observed.

Human estrogen receptor isoform conformational status and ligand induced changes

To determine the possible conformational status of variant hERβ isoforms, a previously used limited trypsin digestion assay (Beekman *et al.* 1993, McDonnell *et al.* 1995) was employed to compare the proteolytic digestion patterns of variant ^{35}S-methionine labeled hERα, hERβ1, hERβ2 and hERβ5 in the presence and absence of estrogens and antiestrogens (4-OH-TAM and LY117018). The results are shown in Fig. 3. In the absence of any ligand, all ER isoforms were sensitive to

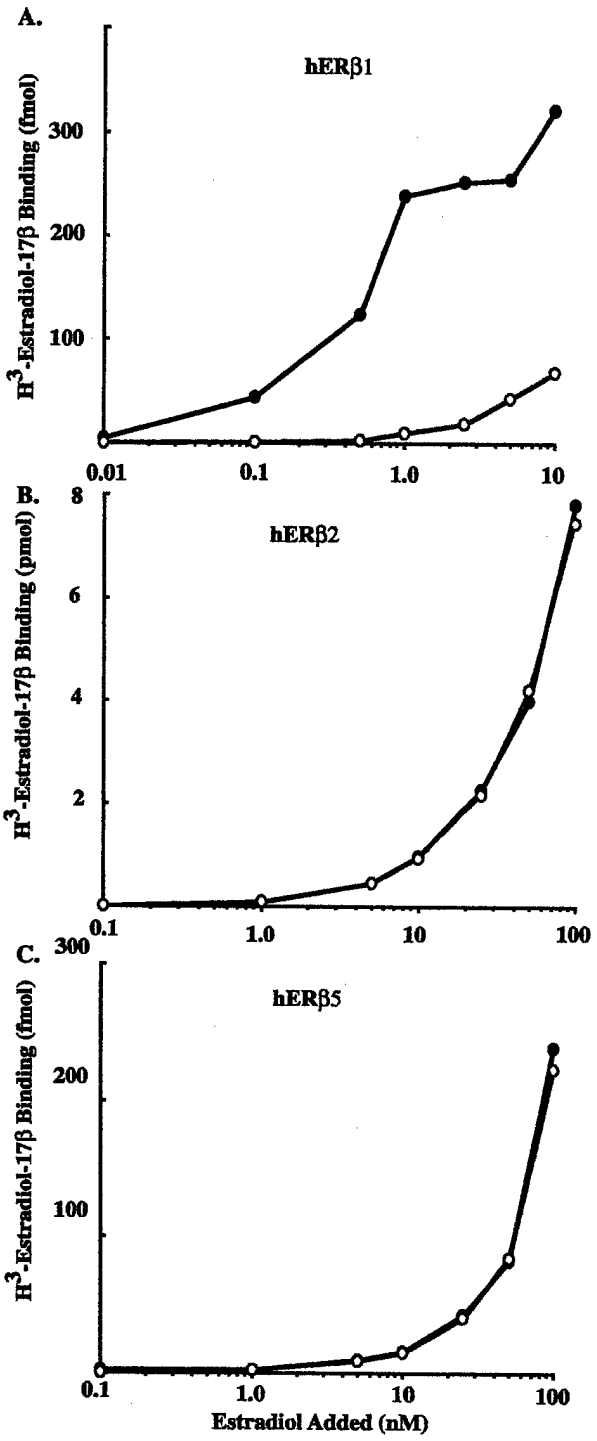


Figure 2 Determination of estradiol binding to (A) hERβ1, (B) hERβ2, and (C) hERβ5. Increasing amounts of [^{3}H]estradiol-17β (0·01–100 nM) were incubated with a constant amount of each *in vitro*-transcribed/translated hERβ protein. Two separate experiments were performed. Total binding is shown by the closed circles and non-specific binding is shown by the open circles.

proteolysis. In the presence of estradiol both hERα and hERβ1 become more resistant to digestion, and a 32·5 kDa resistant band (shown by asterisks in Fig. 3) was observed. In contrast addition of the antiestrogens 4-OH-TAM and LY117018 did not significantly affect the sensitivity of the receptors to trypsin compared with the receptors in the absence of ligand. The sensitivity of the variant isoforms hERβ2 and hERβ5 was not affected by ligand, consistent with their inability to bind ligand and suggesting that the variant isoforms are unlikely to be in an activated conformation.

DNA binding and bending activity of hERβ1 and variant isoforms hERβ2 and hERβ5 proteins

Similar amounts of each recombinantly produced ER isoform protein, determined as described in the Materials and methods section, were used in the electrophoretic mobility shift assays. As previously demonstrated hERβ1 and hERβ2 can bind to an ERE in a gel mobility shift assay (Fig. 4A), although the efficiency of hERβ2 DNA binding was less than hERβ1 (Moore *et al.* 1998). In contrast, Ogawa *et al.* (1998*b*) showed no DNA binding activity for hERβ2. hERβ5 also has the ability to bind an ERE in gel mobility shift assays (Fig. 4A), but was less efficient than hERβ2. The specificity of the binding was determined by competition with excess unlabeled ERE whereas no competition was seen with an excess of unlabeled nonspecific 33 mer oligonucleotide.

DNA bending assays demonstrated that hERα, hERβ1 and hERβ2 were all able to bend DNA as demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison with the mobility of complexes when the ERE is at the end (E) of the DNA fragment (Fig. 4B and C; Nardulli & Shapiro 1993, Lu *et al.* 2000). The calculated bending angle for hERα was 64·8 ± 1 (mean ± S.E.M, n=3), for hERβ1 it was 53·6 ± 0·5 and for hERβ2 it was 54·6 ± 0·7. A lower overall signal of the retarded complexes was seen with hERβ1 and hERβ2 compared with hERα which likely reflects the lower efficiency of the hERβ isoforms of binding to an ERE compared with the hERα. Furthermore, the DNA binding ability of hERβ5 was too low to obtain accurate data for DNA bending calculations. No effect of ligand was observed (data not shown).

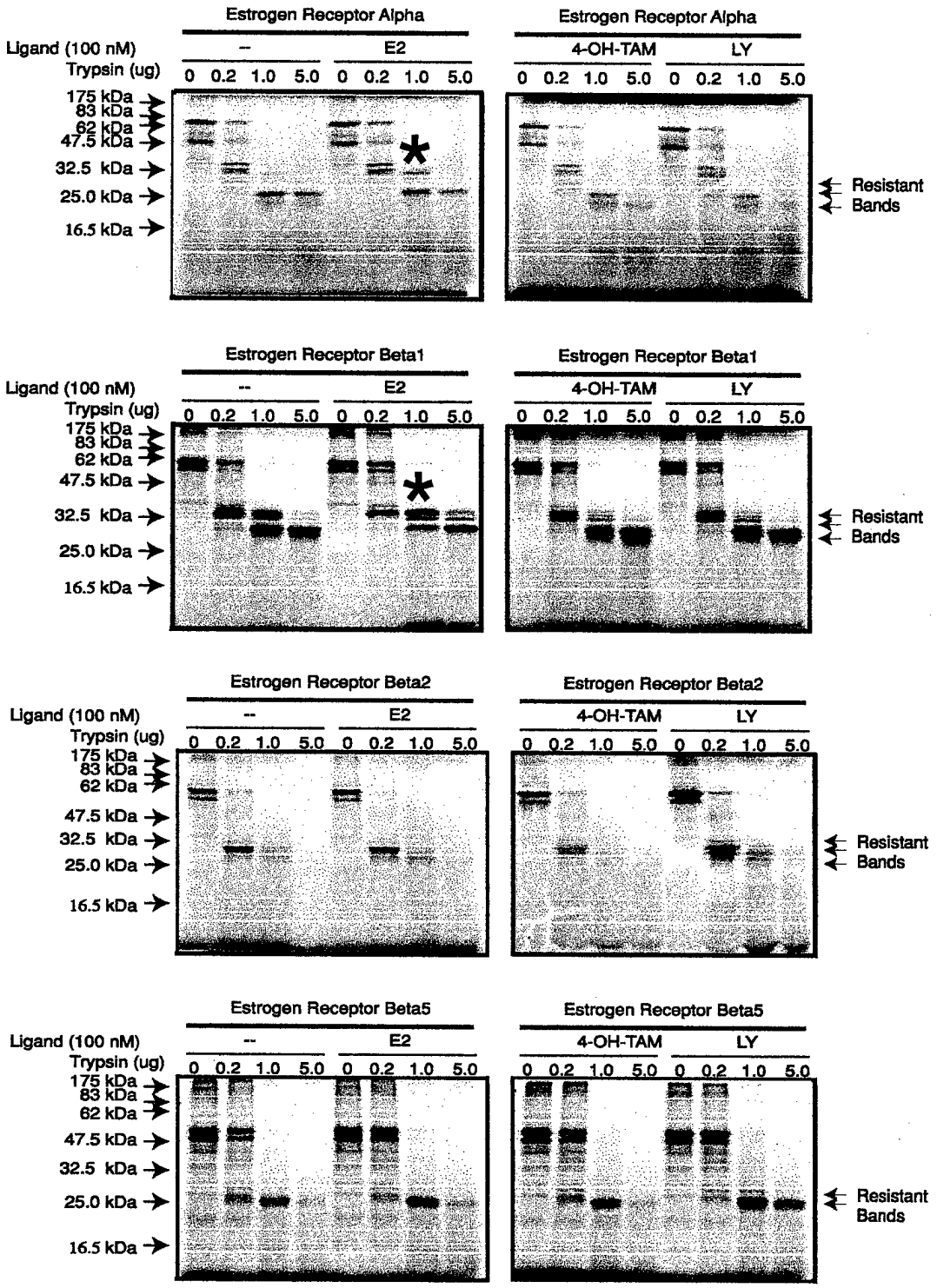
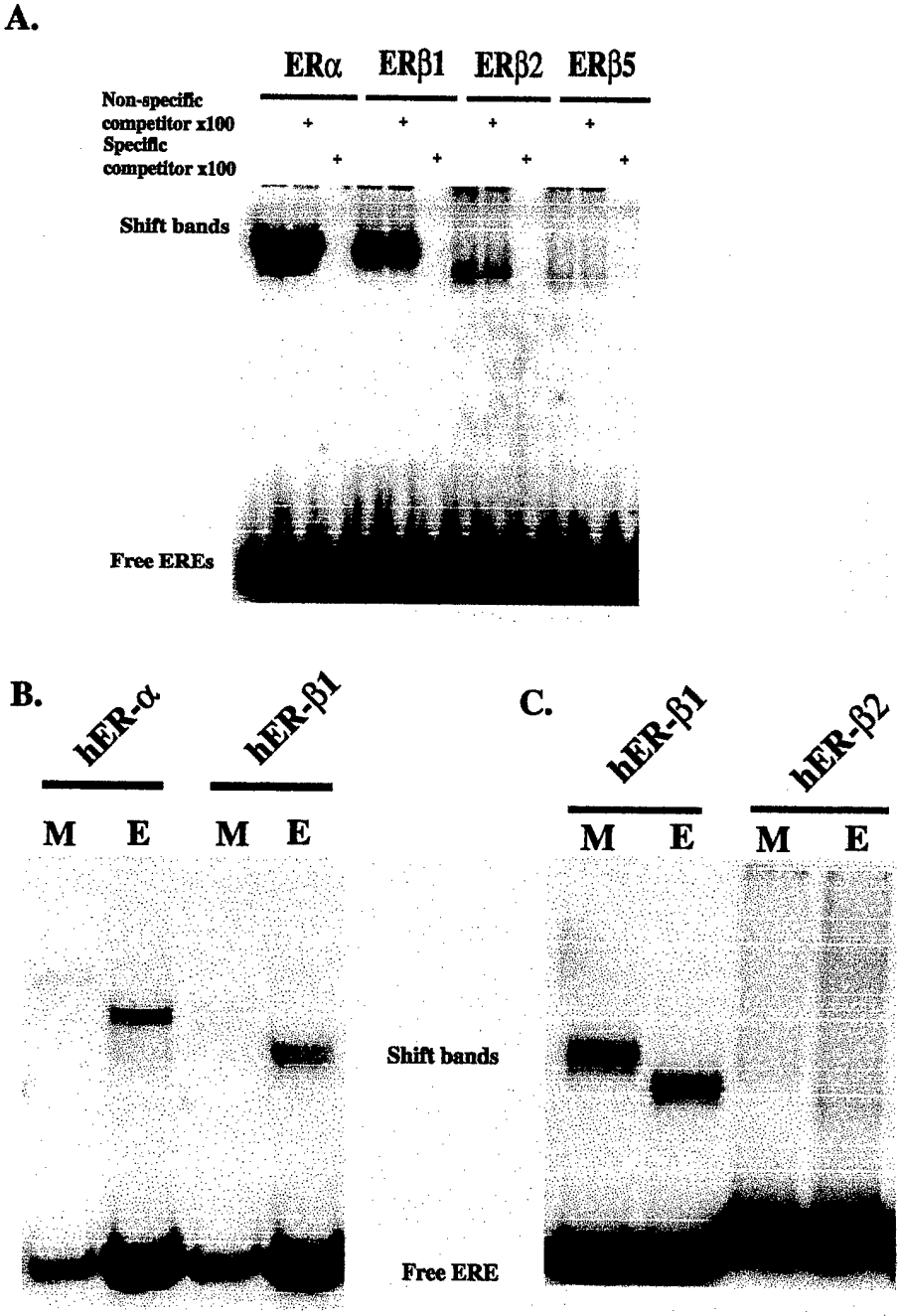


Figure 3 Sensitivity of human estrogen receptor isoforms to protease digestion. Radiolabeled ER was made *in vitro* as described in the Materials and methods section, and digested with increasing levels of trypsin, with and without ligand (E_2, 4-OH-TAM or LY117018 (LY)). The products were visualized by autoradiography after SDS-PAGE. Resistant bands are shown by arrows. The asterisks show the agonist induced resistant 32·5 kDa bands.

Transcriptional activity of hERβ1, hERβ2 and hERβ5

The ability of tagged ERs to activate transcription was initially investigated using Cos-1 cells and an ERE containing reporter gene, ERE_{45}2-delta-TCO-CAT, which has two vitellogenin A2 (−333/−288) EREs upstream of a CAT reporter (Webb *et al.* 1995). Epitope tagged receptors were used so that relative expression of all the ERs could be measured using antibodies to the epitope tag, and preliminary experiments demonstrated that the tagged ERα and ERβ1 were similar to their untagged counterparts in activating transcription with and without ligand (data not shown). Preliminary studies showed that transfection of



50 ng ERα expression vector gave maximal estradiol-induced transactivation of this reporter gene. hERα and hERβ1 activated transcription in a ligand inducible manner (Fig. 5), but hERβ1 was overall less active than hERα ($P<0{\cdot}0001$, $n=5$), and increased expression of hERβ1 did not alter this relationship. These data are consistent with previous findings. As shown in Fig. 5, low doses of estradiol (0·1 nM) which significantly activated hERα did not activate hERβ1 ($P<0{\cdot}0001$, $n=5$), and the apparent ligand-independent activity (zero ligand added) of hERα was significantly higher than that of hERβ1 ($P=0{\cdot}024$, $n=5$). This ligand-independent activity was inhibited by 0·1 and 100 nM of the antiestrogen LY117018 (a raloxifene analog) as well as by hERβ1 (50–450 ng) and hERβ2 (450 ng) (data not shown). These results are not due to over-expression of hERβ1 protein relative to hERα, since under conditions where similar levels of hERα protein (50 ng hERα expression plasmid; see Fig. 6, lane 4) and hERβ1 are expressed (150 ng hERβ1 expression plasmid; see Fig. 6, lane 4) the ligand-independent activity of the receptors is still significantly different (see Fig. 5, compare histogram bar 1 with 7) and the estrogen-inducible (0·1 and 100 nM) activity of hERβ1 (see Fig. 5, compare histogram bars 2 and 3 with histogram bars 8 and 9) is not further increased. The expression of the variant isoforms hERβ2 and hERβ5 alone demonstrated little if any transcriptional activity under these conditions (Fig. 5).

ERα and hERβ isoforms can heterodimerize (Cowley *et al.* 1997), which may underlie the functional interactions between ER isoforms. All hERβ isoforms tested inhibited the transcriptional activity of hERα on an ERE containing promoter (Fig. 7A and B) but the various hERβ isoforms had different efficiencies with hERβ1>hERβ2>hERβ5. Ligand activation of hERβ1 did not affect its ability to decrease the activity of hERα, since under conditions when it was not activated (0 or 0·1 nM estradiol, see Fig. 5) hERβ1 activity was similar to that under conditions when it was activated (100 nM estradiol). Variant isoforms of hERβ had little if any effect on hERβ1 activity on ERE-containing promoters (data not shown).

The transcriptional activity of ER isoforms was next examined on the non-ERE-containing promoter, TGFβ-3-CAT, where the DNA binding domain of ERα is not required for activity (Yang *et al.* 1996). This promoter was shown to be preferentially activated by the raloxifene-bound hERα compared with estradiol in cultured cells (Yang *et al.* 1996), and we have previously shown differential abilities of murine ERβ isoforms to affect this promoter compared with ERE-containing promoters (Lu *et al.* 2000). Therefore, the activity of hERβ isoforms on TGFβ-3-CAT was examined (Fig. 8). Optimal activity for hERα was obtained with transfection of 50 ng expression plasmid (data not shown). A significant increase in transcription was obtained with 0·1 nM LY117018 that was not further increased with 100 nM LY117018 treatment ($P=0{\cdot}0061$, $n=3$). LY117018 significantly increased the transcriptional activity of hERβ1 on the TGFβ-3-CAT reporter gene at the lower levels of hERβ1 expression (50 ng, $P=0{\cdot}008$; 150 ng, $P=0{\cdot}02$, $n=3$) but at high levels of hERβ1 expression (450 ng), a significant increase in ligand-independent activity was seen, and no further increase was seen due to ligand. Overall, hERβ1 was significantly less active than hERα in inducing TGFβ-3-CAT ($P<0{\cdot}0001$, $n=3$).

Figure 4 (A) Determination of the ability of hERα (ERα), hERβ1 (ERβ1), hERβ2 (ERβ2) and hERβ5 (ERβ5) to bind to DNA. Autoradiograph of an electrophoretic mobility gel shift analysis of *in vitro*-transcribed/translated hERα, hERβ1, hERβ2 and hERβ5 proteins binding to a 35 mer double stranded ERE oligonucleotide containing the vitellogenin A2 ERE sequence. Free ERE and the shifted complexes are indicated. The presence of the appropriate ER isoform in the shifted complex was determined by the ability of a specific antibody (H222 for ERα, PA1 for the ERβ proteins) to super-shift the complex (data not shown). Specificity of the complexes was determined by the ability of a 200-fold excess of the unlabeled ERE (specific competitor) to compete for the shifted complex and non-specific interactions were determined using a 200-fold excess of unlabeled nonspecific 33 mer oligonucleotide (nonspecific competitor). (B and C) Comparison of the ability of (B) hERα (hER-α) and hERβ1 (hER-β1) and (C) hERβ1 and hERβ2 (hER-β2) to bend DNA. *In vitro*-transcribed/translated ER isoforms were preincubated with 10 nM estradiol-17β followed by incubation with radiolabeled ERE Bend fragments as described in the Materials and methods and were subjected to electrophoretic gel mobility shift analysis. DNA bending was demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison to the mobility of complexes when the ERE is at the end of the DNA fragment (E).

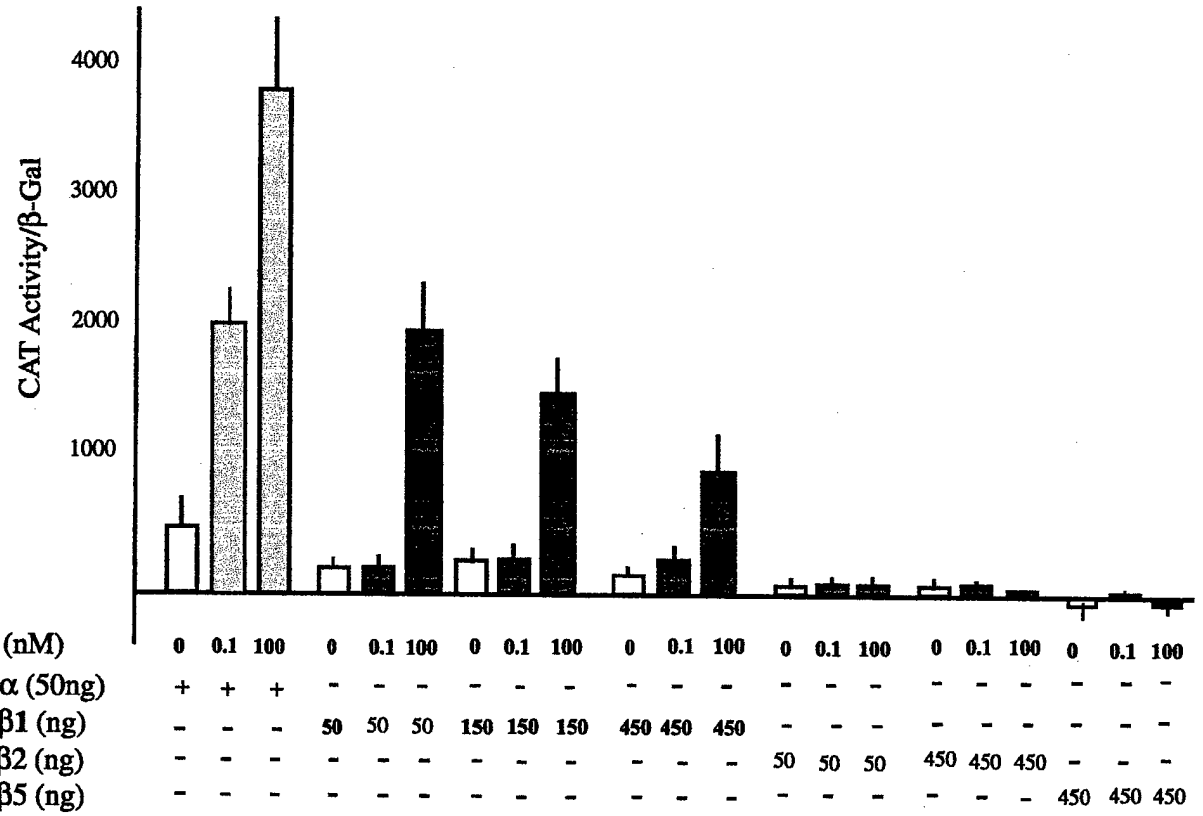


Figure 5 Transcriptional activity of hERα (ERα), hERβ1 (ERβ1), hERβ2 (ERβ2) and hERβ5 (ERβ5) on ERE regulated CAT reporter gene. Increasing amounts of hER isoform expression vectors were co-transfected with an ERE-CAT reporter gene into Cos-1 cells. The transfected cells were treated for 48 h with or without estradiol-17β. Results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency) ± S.E.M. of 5 independent experiments for hERα, hERβ1 and hERβ2, and 3 independent experiments for hERβ5. See text for statistical analysis.

Although there was a trend towards inhibition of TGFβ-3-CAT with increasing expression of hERβ2 or hERβ5 (data not shown), this was not statistically significant.

When the ability of hERβ isoforms to affect hERα activity was investigated at the TGF-β3 promoter, differences between the wild-type and variant isoforms were observed. The wild-type hERβ1 did not significantly affect hERα transcriptional activity at any level of expression tested (Fig. 9A and B). However, under the same conditions hERβ2 significantly inhibited hERα transcriptional activity on TGFβ-3-CAT (P=0·0002, n=3), and as expected the effect was not influenced by LY117018, since hERβ2 does not bind ligand. However, hERβ2 inhibits both the ligand activated and the non-ligand activated (data not shown) hERα (P=0·017, n=3) at the TGFβ-3-CAT promoter. hERβ5 also inhibited hERα transcriptional activity on TGFβ-3-CAT but only at the highest expression of hERβ5 (Fig. 9B, P=0·038, n=3). Similar to their action at an ERE-containing promoter, the truncated ERβ variants ERβ2 and ERβ5 do not modulate wild-type hERβ1 transcriptional activity on TGFβ-3-CAT (data not shown).

Discussion

There is a growing body of evidence that ERα and ERβ can be expressed together in some cell types and independently expressed in others (Dotzlaw *et al.* 1997, Jarvinen *et al.* 2000, Saji *et al.* 2000). If expressed together they form heterodimers, which under experimental conditions are preferred over homodimerization (Cowley *et al.* 1997). Further, transient coexpression of ERα and ERβ in cell lines results in ERβ1-induced reduction of ERα activity

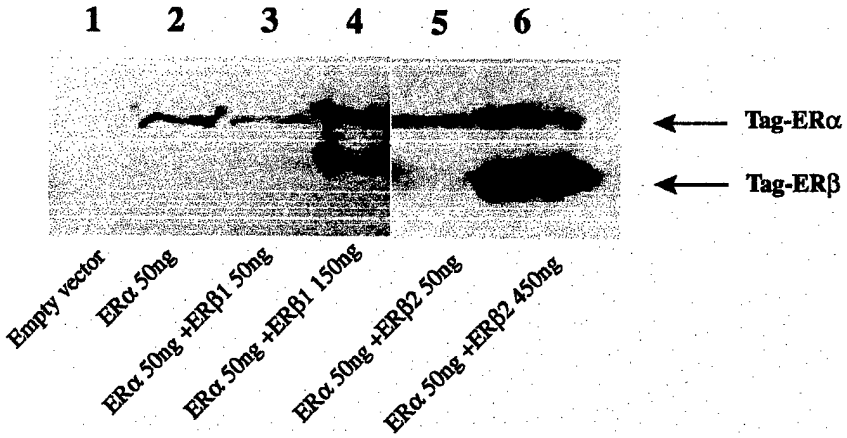


Figure 6 Western blot analysis of Cos-1 cell extracts 48 h after transfection of the indicated amounts of tagged-hERα (ERα), -hERβ1 (ERβ1) and -hERβ2 (ERβ2) expression vectors, as detailed in Materials and methods. Lane 1, extract of Cos-1 cells transfected with empty expression vector alone; lane 2, tagged-hERα expression vector; lane 3, tagged-hERα (50 ng) + tagged-hERβ1 (50 ng) expression vectors; lane 4, tagged-hERα (50 ng) + tagged-hERβ1 (150 ng) expression vector; lane 5, tagged-hERα (50 ng) + tagged-hERβ2 (50 ng) expression vectors; lane 6, tagged-hERα (50 ng) + tagged-hERβ2 (450 ng) expression vectors. The tagged proteins were visualized with anti-Xpress antibody as described in Materials and methods.

at low ligand concentrations, as measured using ERE-regulated reporters (Hall & McDonnell 1999). A conclusion from these data is that ERβ can directly modulate ERα activity. This has significance since many reports exist of differential expression of the two receptors under conditions of altered estrogen sensitivity. For example, ERβ expression is significantly downregulated and ERα expression upregulated during human breast tumorigenesis, suggesting that ERβ's ability to modulate ERα is significantly altered during breast tumorigenesis (Leygue *et al.* 1998*b*, Roger *et al.* 2001). In addition, current data show that in normal and neoplastic breast tissues, the level of expression of the C-terminally truncated ERβ variants, ERβ2 and ERβ5, is markedly higher than the ligand binding ERβ1. These data suggest that the variant ERβ isoforms may also have a role in modulating estrogen and possibly antiestrogen action in human breast cells. The experiments described in this manuscript were undertaken to gain insight into the possible role of the truncated ERβ variants.

Our data show that only hERβ1 is able to bind ligand. Steroid hormone receptors are known to undergo conformational changes during the process of activation especially due to ligand binding, and differences are seen between agonist and antagonist binding (Beekman *et al.* 1993, McDonnell *et al.* 1995). Recent structural analyses of the ligand binding domain (LBD) of several nuclear receptors suggest that the LBD contains common structural motifs that generate a conserved ligand binding pocket, and that agonists and antagonists bind to the same site but induce different conformational changes that are now known to affect transcriptional function, providing structural evidence for antagonism (Brzozowski *et al.* 1997). The variant hERβ isoforms, while not binding ligand, may exist in an activated state in the absence and presence of ligand; however, our data suggest that hERβ2 and hERβ5 are unlikely to be in an activated conformation, and this is consistent with their inability to activate transcription of either a 'classical' or a 'non-classical' estrogen receptor regulated reporter gene.

All ERβ isoforms examined (ERβ1, ERβ2, and ERβ5) inhibit the transcriptional activity of ERα on ERE-containing promoters, while only ERβ1 has any activity alone. This confirms and extends previous data and demonstrates that the relative inhibitory activity of the ERβ isoforms is

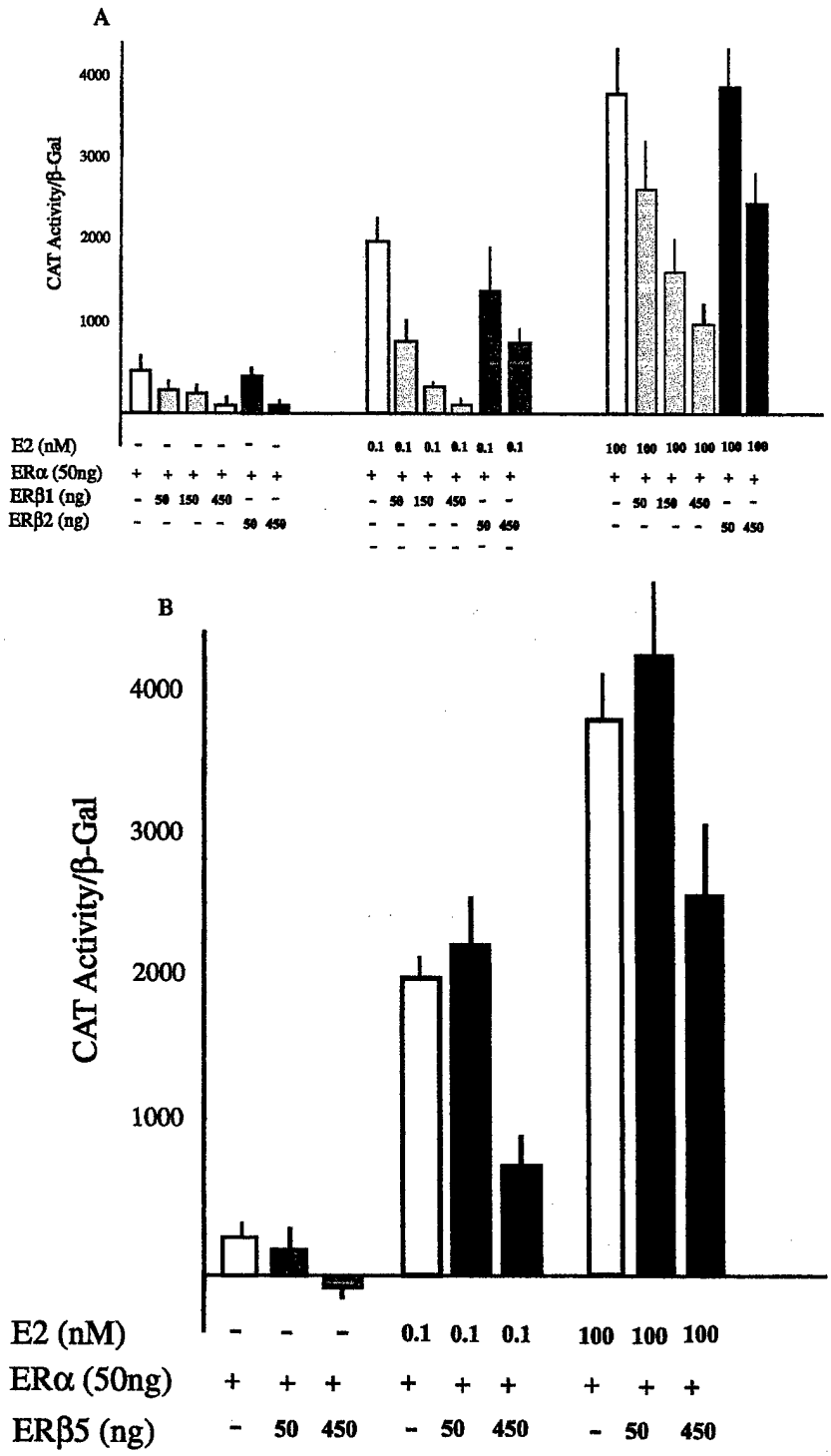


Figure 7 Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from an ERE (vitellogenin A2) regulated CAT reporter gene in the presence and absence of ligand following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency) ± S.E.M. of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.

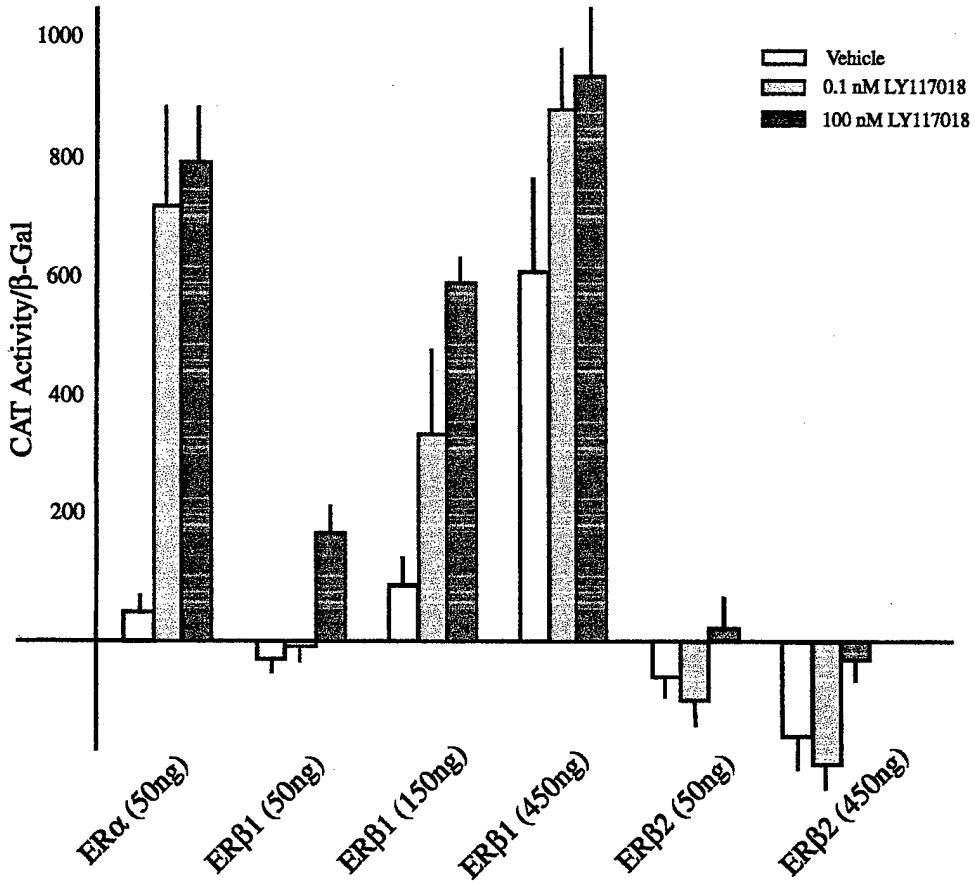
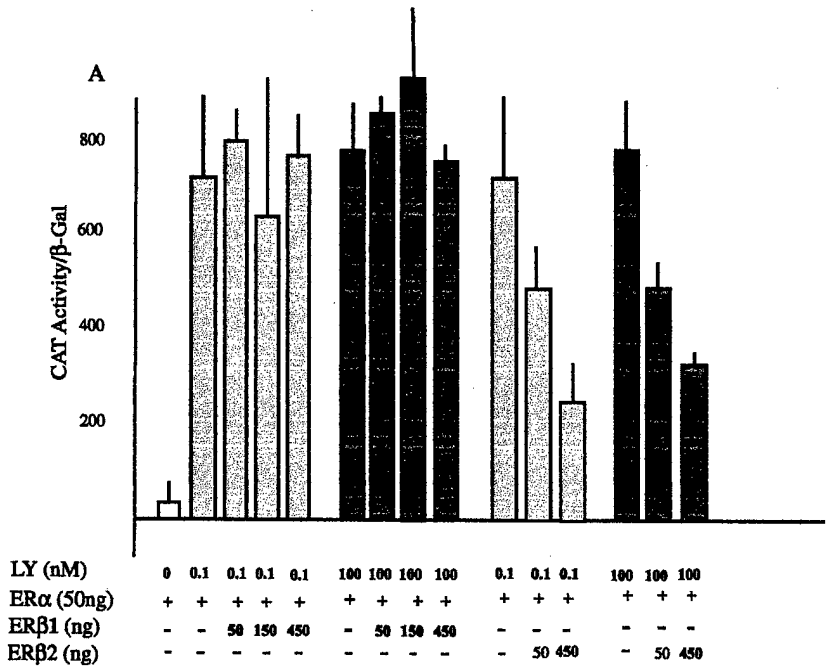


Figure 8 Transcriptional activity of hERα (ERα), hERβ1 (ERβ1) and hERβ2 (ERβ2) on a novel raloxifene responsive element regulated reporter gene, TGF-β3-CAT. Increasing amounts of hER isoform expression vectors were co-transfected with a TGF-β3-CAT reporter gene into Cos-1 cells. The transfected cells were treated for 48 h with or without the raloxifene analog LY117018. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)±S.E.M. of 3 independent experiments. See text for statistical analysis.

ERβ1>ERβ2>ERβ5. This correlates with the relative efficiencies with which ERβ homodimers bind to DNA and may suggest a competition of the beta isoform homodimers with ERα homodimers for DNA binding. However, since heterodimers are preferred under these conditions, it is likely that these predominate under our experimental conditions and the intrinsically lower transcriptional activity of the heterodimers are predominant. Cowley *et al.* (1997) demonstrated that when hERα and hERβ1 are expressed at both a 1:1 and 1:2 ratio the ERα/ERβ1 heterodimer was predominant. This heterodimer had a DNA binding affinity similar to that of the ERα homodimer, and was capable of recruiting steroid receptor coactivator-1 (SRC-1). However, the heterodimer has less transcriptional activity than the ERα/ERα homodimer, suggesting that it may be less efficient in recruiting coactivators than the ERα homodimer. In contrast, the C-terminally truncated hERβ2 has markedly reduced ability to bind to DNA and likely the ERα/ERβ2 heterodimer also binds less well than ERα/ERα homodimers to an ERE (Moore *et al.* 1998, Ogawa *et al.* 1998*b*). But in contrast to hERβ1, hERβ2 does not recruit coactivators (Ogawa *et al.* 1998*b*). Our data show that hERβ5 is less efficient than hERβ2 in binding to DNA, and is also unlikely to recruit coactivators. However, at an ERE the wild-type hERβ1 is more potent than either of the two variants in inhibiting the ability of ERα to activate transcription. So it appears that the inability to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CAT Activity/β-Gal
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600
400
200
LY (nM) 0 0.1 0.1 0.1 0.1 100 100 100 100 0.1 0.1 0.1 100 100 100
ERα (50ng) + + + + + + + + + + + + + + +
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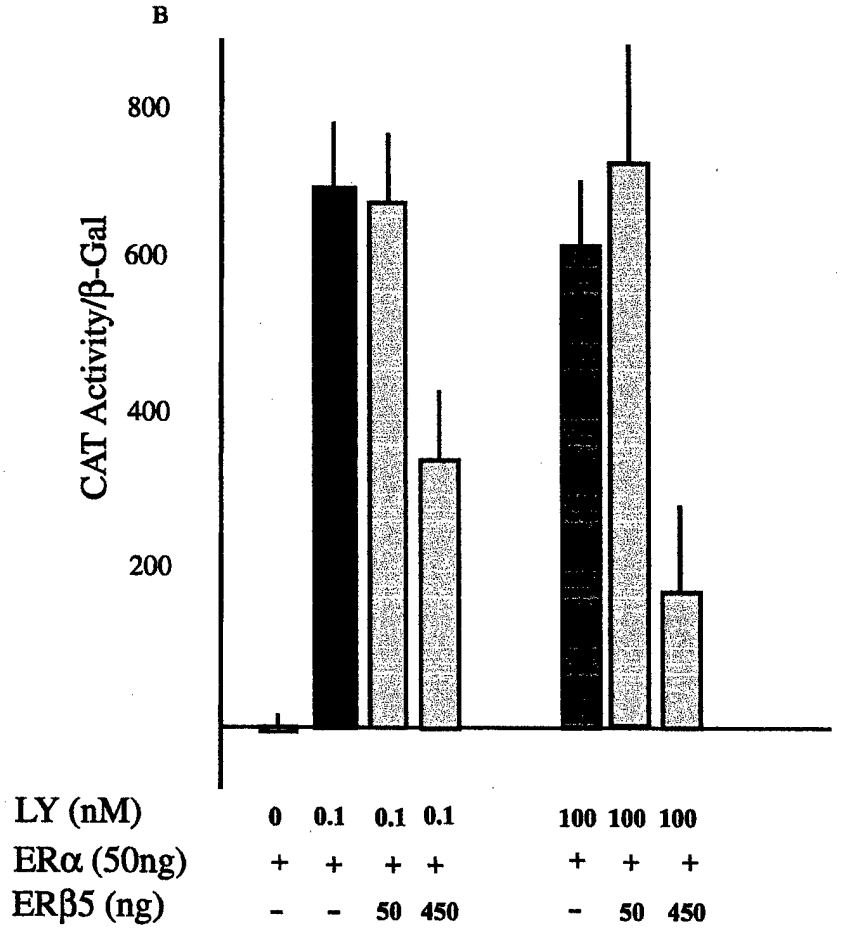
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recruit coactivators is not correlated with the ability of ERβ isoforms to inhibit ERα activity. Since DNA activity is also a reflection of efficiency of dimerization, it is speculated that the truncated ERβ isoforms have reduced ability to dimerize with ERα and form stable heterodimers than the wild-type ERβ1. Together with our Western blot data it seems that significant inhibition of ERα transcription occurs at levels of ERβ1 expression that are less than or equivalent to ERα (50 ng ERβ1 plasmid makes less protein than 50 ng ERα plasmid, but still significantly affects ERα transcription activity). Therefore our data would be consistent with the mechanism of inhibition being related to a high efficiency of dimerization and reduced efficiency in recruiting coactivators, but not the inability to recruit coactivators.

Interestingly, marked differences in the ability of the ERβ isoforms to affect ERα activity are seen at an estrogen receptor responsive site where the mechanism of transcriptional regulation is quite distinct from that operating at a classical ERE, e.g. the so-called raloxifene responsive element in the TGF-β3 promoter (Yang *et al.* 1996). This is in marked contrast to the results seen at an ERE regulated reporter gene. The ER responsive site in the TGF-β3 promoter is poorly activated by the estradiol–ERα complex, but is strongly activated by the raloxifene–ERα complex. In addition, the DNA binding domain of the ER is not required for this activation. It is assumed that protein–protein interactions between ERα and other transcription factors bound to this promoter are involved in regulation. However, the identity of these 'other' transcription factors is unknown. Using an analog of raloxifene, LY117018 (Lu *et al.* 2000), we have confirmed that this promoter is poorly activated by the estradiol–ERα complex (and this was not altered in our hands by treatment of the transfected Cos-1 cells with epidermal growth factor (Lu & Giguere 2001); data not shown) but was significantly activated by the LY117018–ERα complex. Similarly, the LY117018–hERβ1 complex was found to activate transcription from the TGF-β3 promoter, but in contrast to the murine ERβ1 (Lu *et al.* 2000), is less active than the LY117018–hERα complex. Human ERβ2 and hERβ5 alone could not activate this promoter. This is in contrast to the murine ERβ2 variant (Lu *et al.* 2000) which is structurally quite different to the hERβ2. Furthermore, no murine equivalent to either hERβ2 or hERβ5 isoforms, that are frequently expressed in human tissues, has as yet been identified. However, coexpression of increasing amounts of hERβ2 and hERβ5 with ERα resulted in inhibition of LY117018–ERα transcriptional activity but not LY117018–ERβ1 activity from the TGF-β3 promoter. In contrast to an ERE-containing promoter is the observation that the wild-type hERβ1 did not significantly inhibit the transcriptional activity of the LY117018–ERα complex at the TGF-β3 promoter. At this promoter the differences in the hERβ isoform activity on LY117018–ERα complexes were correlated to the ability to recruit coregulatory factors. Significant effects of hERβ2 on hERα were seen under conditions of equimolar expression, as determined by Western blot analysis of the similarly tagged proteins, but hERβ5 was less active than hERβ2 and this is consistent with a reduced efficiency of dimerization. There appears to be differential expression of hERβ isoforms at least at the RNA level in different human tissues, as well as altered relative expression during breast tumorigenesis (Leygue *et al.* 1999, Omoto *et al.* 2002), and altered levels of hERβcx (hERβ2) as well as hERβ1 during prostate cancer progression (Fujimura *et al.* 2001). Therefore, it is possible that the differential activities of hERβ isoforms on some genes may have both physiological and pathophysiological importance.

In conclusion we have characterized some potential functions of several commonly expressed hERβ isoforms. Generally, the ligand binding wild-type hERβ1 has transcriptional activity alone on both 'classical' and 'non-classical' estrogen responsive promoters, although it is less efficient than ERα. Furthermore, the hERβ family of receptors generally negatively modulate ERα

Figure 9 Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from a TGF-β3-CAT reporter gene in the presence and absence of the raloxifene analog LY117018 following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)±S.E.M. of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.

transcriptional activity when coexpressed at 'classical' as well as 'non-classical' ER responsive promoters. However, promoter specific differential activity of the various hERβ isoforms was found, in particular between the wild-type hERβ1 and its C-terminally truncated variants hERβ2 and hERβ5. The possibility that there is differential expression of the hERβ isoforms suggests that they may have a role in differentially modulating estrogen action.

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Relationship of coregulator and oestrogen receptor isoform expression to *de novo* tamoxifen resistance in human breast cancer

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This study addresses the hypothesis that altered expression of oestrogen receptor-beta and/or altered relative expression of coactivators and corepressors of oestrogen receptors are associated with and may be mechanisms of *de novo* tamoxifen resistance in oestrogen receptor positive breast cancer. All cases were oestrogen receptor +, node negative, primary breast tumours from patients who later had no disease progression (tamoxifen sensitive) or whose disease progressed while on tamoxifen (tamoxifen resistant). Using an antibody to oestrogen receptor-beta that detects multiple forms of this protein (total) but not an antibody that detects only full-length oestrogen receptor-beta 1, it was found that high total oestrogen receptor beta protein expressors were more frequently observed in tamoxifen sensitive tumours than resistant tumours (Fisher's exact test, P=0.046). However, no significant differences in the relative expression of oestrogen receptor β2, oestrogen receptor β5 and full-length oestrogen receptor β1 RNA in the tamoxifen sensitive and resistant groups were found. Also, when the relative expression of two known coactivators, steroid receptor RNA activator and amplified in breast cancer 1 RNA to the known corepressor, repressor of oestrogen receptor activity RNA, was examined, no significant differences between the tamoxifen sensitive and resistant groups were found. Altogether, there is little evidence for altered coregulators expression in breast tumours that are *de novo* tamoxifen resistant. However, our data provide preliminary evidence that the expression of oestrogen receptor β protein isoforms may differ in primary tumours of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy.
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The ability of anti-oestrogens such as tamoxifen to compete with oestrogens for binding to OR and to antagonise their mitogenic action provides the basic rationale for endocrine therapy and prevention (for a review see (Osborne, 1998b) in breast cancer. Adjuvant tamoxifen post-operative therapy reduces the number of recurrences and prolongs survival in women whose primary tumours are oestrogen receptor (OR) positive (Group, 1998). However, even though OR level is considered a marker for predicting the likelihood of responding to adjuvant hormonal therapies, some patients, whose primary tumours are OR positive do not respond to tamoxifen treatment. Such apparent *de novo* tamoxifen resistance does not depend upon the level of OR within the primary tumour. As well many of those patients whose disease initially responds to tamoxifen, progress while still under treatment having acquired resistance and this occurs despite continued expression of OR. Thus suggesting other components of the oestrogen signalling pathway may be altered. Recent observations using laboratory models (Hall and McDonnell, 1999; Lanz *et al*, 1999; McKenna *et al*, 1999; Montano *et al*, 1999) have demonstrated that altered levels of OR isoforms and/or alteration of expression of coactivators and corepressors can deregulate oestrogen and antioestrogen activity in target cells, suggesting the hypothesis that altered levels of OR isoforms and/or coregulators *in vivo* could be a mechanism of tamoxifen resistance. Previously we have demonstrated that the relative expression of ORα/ORβ as well as the relative expression of some OR coactivators to corepressors is significantly altered during breast tumourigenesis *in vivo* (Leygue *et al*, 1998; Murphy *et al*, 2000). Furthermore, since these alterations parallel the marked changes in oestrogen action that accompany breast tumourigenesis, they may have a role in this process. To explore the hypothesis that such changes could underlie *de novo* tamoxifen resistance *in vivo*, the expression of OR isoforms, two known coactivators (steroid receptor RNA activator (SRA), (Lanz *et al*, 1999) and amplified in breast cancer-1 (AIB1) (Anzick *et al*, 1997)) and one corepressor (repressor of oestrogen receptor activity, repressor of oestrogen receptor activity (ROA) (Montano *et al*, 1999)) of OR activity have been investigated in primary breast tumours from node negative patients whose tumours were OR

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positive and that subsequently responded or had disease progression while on adjuvant tamoxifen therapy.

MATERIALS AND METHODS

Human breast tumours

All breast tumour cases used for this study were selected from the NCIC-Manitoba Breast Tumour Bank (Winnipeg, Manitoba, Canada). As previously described (Hiller *et al*, 1996), tissues are accrued to the Bank from cases at multiple centres within Manitoba, rapidly collected and processed to create matched formalin-fixed-embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by coloured inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in Hematoxylin/Eosin (H&E) stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks from cases for this study. For each case, interpretations included an estimate of the cellular composition (including the percentage of invasive epithelial tumour cells and stroma), tumour type and tumour grade (Nottingham score). Steroid receptor status was determined for all cases by ligand binding assay performed on an adjacent portion of tumour tissue. Tumours with oestrogen receptor levels above 3 fmol mg^{-1} of total protein were considered OR positive.

To identify cases that responded divergently to tamoxifen, review of approximately 1000 consecutive cases was undertaken to identify cases that were OR positive, node negative and that had been treated with adjuvant tamoxifen following surgery +/− local radiation. From these the first cohort of 27 cases was selected to include a subset (n=13) that had shown progression of disease (either died or alive with recurrent disease, referred to as tamoxifen resistant cases) and a similar control subset (n=14) specifically selected to comprise cases with similar lengths of follow-up, OR status, tumour grade and tumour histology, but that had shown no progression of disease (referred to as tamoxifen sensitive cases). The tumour characteristics were: (1) 'Tam Sensitive' group median OR was 60.5 fmol mg^{-1} protein (range 6–146 fmol mg^{-1} protein), median PR was 32 fmol mg^{-1} protein (range 8–216 fmol mg^{-1} protein); median grade was five (range 4–8); median age at biopsy was 69 years (range 35–87 years); median follow-up time was 56 months (range 18–79); (2) 'Tam Resistant' group median OR was 57 fmol mg^{-1} protein (range 4–136 fmol mg^{-1} protein); median PR was 14 fmol mg^{-1} protein (range 4–288 fmol mg^{-1} protein); median grade was six (range 4–9); median age at biopsy was 67 years (range 49–83 years); median follow-up time was 56 months (range 9–85).

For the RNA studies, frozen tissue corresponding to the blocks for several of the first cohort of older cases used above, were not available. Therefore, after further review of the tumour bank as described above, a second study cohort was selected that also had frozen tissue available. The relevant patient/tumour characteristics were similar to the above cohort, although the follow-up time was shorter: (1) 'Tam Sensitive' group (n=16) median OR was 37.5 fmol mg^{-1} protein (range 4.4–146 fmol mg^{-1} protein), median PR was 44 fmol mg^{-1} protein (range 13.1–216 fmol mg^{-1} protein); median grade was six (range 4–9); median age at biopsy was 72 years (range 47–87 years); median follow-up time was 39 months (range 13–76); (2) 'Tam Resistant' group (n=16) median OR was 21.5 fmol mg^{-1} protein (range 5.6–107 fmol mg^{-1} protein); median PR was 14.3 fmol mg^{-1} protein (range 7.8–288 fmol mg^{-1} protein); median grade was six (range 4–9); median age at biopsy was 71 years (range 60–89 years); median follow-up time was 34 months (range 9–63).

Immunohistochemistry

Immunohistochemistry was performed on serial 5 μm sections from a representative, formalin fixed paraffin embedded archival tissue block from each tumour. Immunohistochemical staining for ORβ was performed using two different primary antibodies. IgYERB503 (a gift from Dr Jan-Ake Gustafson) detects total ORβ isoforms (Horvath *et al*, 2001; Saji *et al*, 2000) and GC17 (a gift from Dr Shuk-Mei Ho) detects only the full-length ORβ (Leav *et al*, 2001). The GC17 polyclonal antibody was raised in rabbits against a peptide sequence in the F domain of the human OR-β receptor (amino acids 449 to 465) and its specificity validated previously (Leav *et al*, 2001). The epitope to which the IgYERB503 antibody is directed is not known, but this polyclonal chicken antibody was raised to an ORβ recombinant protein which was disrupted in the ligand binding domain by insertion of 18 additional amino acids, but was subsequently shown to also recognise the full-length non-inserted ORβ protein (Saji *et al*, 2000). Antibodies were applied using an automated tissue immunostainer (Discovery module, Ventana Medical Systems, Phoenix, AZ, USA), DAB immunohistochemistry kit and bulk reagents that were supplied by the manufacturer. Briefly, the Discovery staining protocol was set to 'Standard Cell Conditioning' procedure, followed by 12 h incubation with primary antibody and 32 min incubation with secondary antibody. Concentrations of primary antibodies initially applied to the Ventana instrument were 1:200 for IgYERB503 and 1:50 for GC17, which translates into final concentrations of 1:600 and 1:150 after a 1:3 dilution with buffer dispensed onto the slide with the primary antibody. Levels of nuclear ORβ expression were scored semi-quantitatively, under the light microscope. Scores were obtained by estimating average signal intensity (on a scale of 0–300) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an overall IHC-score. Cases with a score lower than or equal to 100 were considered negative or weakly positive, whereas tumours with scores higher than 100 were classified as positive for ORβ expression (Al-Haddad *et al*, 1999).

RNA Extraction and RT–PCR conditions

Total RNA was extracted from 20 μm frozen tissue sections (20 sections per tumour) using Trizol™ reagent (Life Technologies, NY, USA) according to the manufacturer's instructions and quantified spectrophotometrically. One μg of total RNA was reverse transcribed in a final volume of 25 μl as previously described (Leygue *et al*, 1996).

Primers and PCR conditions

Coregulators The primers used were: SRAcoreU primer (5′-AGGAACGCGGCTGGAACGA-3′; sense; positions 35–53, Genbank accession number AF092038) and SRAcoreL primer (5′-AGTCTGGGGAACCGAGGAT-3′; antisense; position 696–678, Genbank accession number AF092038); AIB1-U primer (5′-ATACTTGCTGGATGGTGGACT-3′; sense; positions 110–130, Genbank accession number AF012108) and AIB1-L primer (5′-TCCTTGCTCTTTTATTTG ACG-3′; antisense; positions 458–438, Genbank accession number AF012108); ROA-U primer (5′-CGAAAAATCTCCTCCCCTACA-3′; sense; positions 385–405, Genbank accession number AF150962) and ROA-L primer (5′-CCTGCTTTGCTTTTTCTACCA-3′; antisense; positions 781–761, Genbank accession number AF150962).

Radioactive PCR amplifications for SRA were performed and PCR products analysed as previously described (Leygue *et al*, 1999b) with minor modifications. Briefly, 1 μl of reverse transcrip-

tion mixture was amplified in a final volume of 15 μl, in the presence of 1.5 μCi of (α-^{32}P) dCTP (3000 Ci mmol^{-1}), 4 ng μl^{-1} of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). For SRA each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and exposed 2 h to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA, USA).

PCR amplifications for AIB1 and ROA were performed and PCR products analysed as previously described (Leygue *et al*, 1996) with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 20 μl, in the presence of 4 ng μl^{-1} of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). For AIB1, each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 55°C and 30 s at 72°C). For ROA each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 57°C and 30 s at 72°C). PCR products were then separated on agarose gels stained with ethidium bromide as previously described (Leygue *et al*, 1996).

Primers for OR isoforms

ORα-U primer (5′-TGTGCAATGACTATGCTTCA-3′; sense; located in ORα 792–811) and ORα-L primer (5′-GCTCTT-CCTCCTGTTTTTA-3′; antisense; located in ORα 940–922). Nucleotide positions given correspond to published sequences of the human ORα cDNA (Green *et al*, 1986). PCR amplifications were performed and PCR products analysed as previously described with minor modifications (Dotzlaw *et al*, 1997). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi (α-^{32}P) dCTP (3000 Ci mmol^{-1}), 2 ng μl^{-1} of ORα-U/ORα-L and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C and 30 s at 72°C).

A previously validated triple primer assay was used to determine the relative expression of ORβ1 and its variant isoforms ORβ2 and ORβ5 (Leygue *et al*, 1999a). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi of (α-^{32}P) dCTP (3000 Ci mmol^{-1}), 4 ng μl^{-1} of each primer (ORβ1U, ORβ1L and ORβ2L) and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA).

All OR PCRs consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Three independent PCRs were performed.

Quantification of SRA and OR RNA expression Exposed screens were scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA, USA) and the intensity of the signal corresponding to SRA or the appropriate OR isoform PCR fragments was measured using Quantity One™ software (Bio-Rad, Hercules, CA, USA). Three independent PCRs were performed. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumour measured in each set of PCR experiments (always the same tumour) and all signals were expressed relative to this signal. Levels of SRA was expressed relative to ROA (SRA/ROA), AIB1 (SRA/AIB1) or ORα (SRA/ORα) in each individual tumour sample. Levels of ORβ isoforms were expressed relative to other ORβ isoforms shown under statistical analysis and as previously described (Leygue *et al*, 1999a).

Quantification of the relative expression of the deleted SRA variant RNA It has previously been shown that the coamplification of a full-length and a deleted variant SRA (SRA-Del) cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (Leygue *et al*, 1999b). For each sample, the signal corresponding to the SRA-Del was measured using Quantity One™ software (Bio-Rad, Hercules, CA, USA) and expressed as a percentage of the corresponding core SRA signal. For each case, three independent assays were performed and the mean determined.

Quantification of ROA and AIB1 RNA expression Following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst™ (Bio-Rad, Hercules, CA, USA). At least, three independent PCRs were performed. A value of 1 was arbitrarily assigned to the ROA or AIB1 signal of one particular tumour and is the same tumour as described above and all signals were expressed relative to this signal. Levels of AIB1 were expressed relative to ROA (AIB1/ROA) and ORα (AIB1/ORα), and levels of ROA were expressed relative to ORα (ROA/ORα).

Statistical analysis Differences between tamoxifen sensitive and tamoxifen resistant cases were tested using the Mann–Whitney rank sum test, two-tailed. Potential differences in expression between the two groups with respect to each ORβ isoform RNA relative to other ORβ isoform RNA expression (e.g. log ORβ1/total ORβ; log ORβ2/total ORβ; log ORβ5/total ORβ, as previously described (Leygue *et al*, 1999a)), and the relative expressions of coregulators (i.e. logAIB1/ROA; logSRA/ROA; logSRA/AIB1; logAIB1/ORα; logSRA/ORα; logROA/ORα) were determined.

Tumours were classified as low (scores between 0 and 100) and high (150–300) ORβ expressors, and differences between tamoxifen sensitive and tamoxifen resistant cases were tested using Fisher's exact test. Correlation between ORβ protein expression (IHC-score) and tumour characteristics was tested by calculation of the Spearman coefficient *r*.

RESULTS

Expression of ORβ protein in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment

ORβ protein was determined immunohistochemically on adjacent sections from each tumour, using two different antibodies. GC-17 is an antibody recognizing an epitope in the C-terminus of full-length ORβ1 (Leav *et al*, 2001). Normal breast tissue was used as a positive control and is shown in Figure 1A. Examples of staining in human breast tumour sections are shown in Figure 1B–D. Some tumour sections showed no (Figure 1B, full-length ORβ score=0) or low (Figure 1C, full-length ORβ score=100), while others showed strong full-length ORβ signals (Figure 1D, wild-type ORβ score=300). Tumours were classified as low (scores between 0 and 100) and high (150–300) full-length ORβ protein expressors, and differences between tamoxifen sensitive and resistant tumours determined by Fisher's exact test. No significant differences were found.

IgYERbeta503 is an antibody that recognises ligand binding and non-ligand binding ORβ protein isoforms (Horvath *et al*, 2001; Saji *et al*, 2000) and which we refer to as total ORβ protein. Normal breast tissue was used as a positive control and is shown in Figure 2A. Examples of staining with this antibody in human breast tumour sections are shown in Figure 2B–D. Some sections showed no (Figure 2B, total ORβ score=0) or low (Figure 2C, total ORβ score=100) total ORβ expression whereas others had strong total ORβ protein signal (Figure 2D, total ORβ score=300).

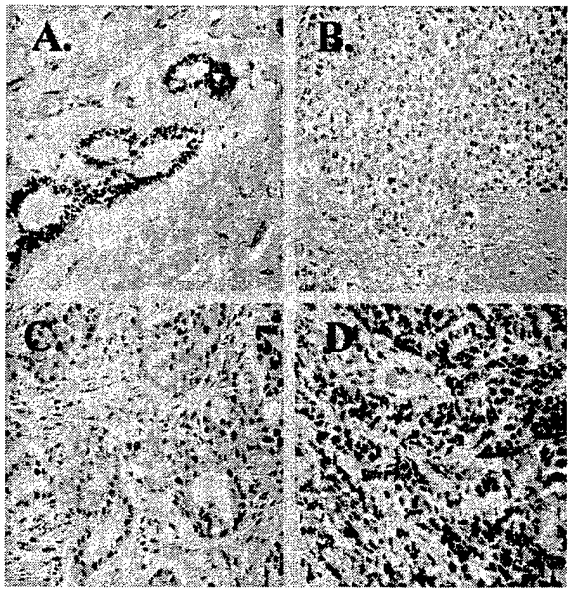

Figure 1 Examples of immunohistochemistry using the GC-17 antibody which only recognises the full-length ORβ1: (**A**) normal human breast tissue; (**B**) ORβ1 negative human breast tumour, H-score=0; (**C**) ORβ1 low expressing human breast tumour, H-score=100; (**D**) ORβ1 high expressing human breast tumour, H-score=150.

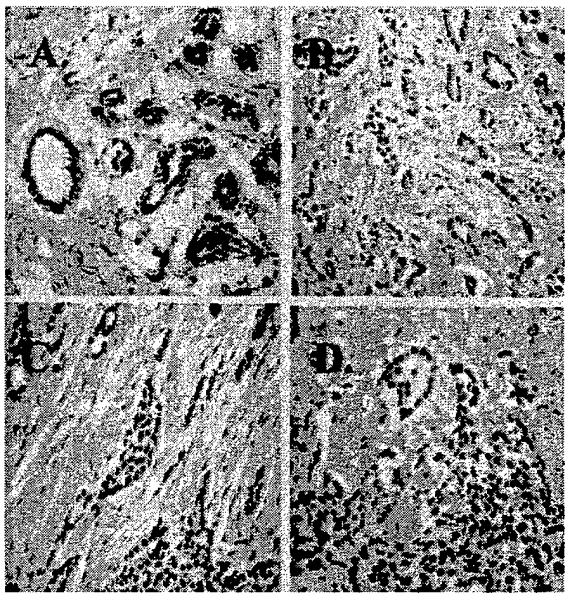

Figure 2 Examples of immunohistochemistry using the IgYERβ503 antibody which recognises most ORβ isoforms: (**A**) normal human breast tissue; (**B**) ORβ negative human breast tumour, H-score=25; (**C**) ORβ low expressing human breast tumour, H-score=100; (**D**) ORβ high expressing human breast tumour, H-score=225.

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Tumours were classified as low and high total ORβ protein expressors, and there was a statistically significant difference in high total ORβ protein between the Tamoxifen sensitive and resistant groups (Fisher's exact test, P=0.046). High total ORβ protein expressors were more frequently observed in tamoxifen sensitive tumours than resistant tumours.

Correlation between ORβ protein expression and tumour characteristics was tested by calculation of the Spearman coefficient. A positive correlation between ORβ1 (GC17) protein and progesterone receptor (PR) levels (Spearman r=0.44, P=0.022) was found when each was examined as continuous variables. When tumours were divided into PR+ (>10 fmol mg^{-1} protein) or PR− ($\leqslant 10$ fmol mg^{-1} protein) groups there was a significantly higher level of ORβ1 (GC17) protein in PR+ tumours compared to PR− tumours (Mann–Whitney test, P=0.0268; median for PR+ tumours=55, range 5–150 and median for PR− tumours=10, range 0–75). As well, there was also a significantly higher level of total ORβ (IgY503) protein in PR+ tumours compared to PR− tumours (Mann–Whitney test, P=0.0085; median for PR+ tumours=125, range 25–270 and median for PR− tumours=50 range 0–100).

Relative expression of ORβ isoform RNA in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on adjuvant tamoxifen To determine if the differences described above in ORβ protein expression were correlated with differences in ORβ variant isoform RNA expression, we compared the relative expression of ORβ variant RNA to full-length ORβ RNA in the tamoxifen sensitive and resistant groups. Unfortunately, frozen tissue samples corresponding to many of the paraffin blocks from patients in the cohort used for immunohistochemistry were not available. Therefore additional cases selected were selected from the tumourbank as described in Materials and Methods. Using previously validated assays (Leygue *et al*, 1998; 1999a) the relative expression of ORβ2, ORβ5 and full-length ORβ1 RNA in the tamoxifen sensitive and resistant groups was not significantly different.

Relative expression of coregulators in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment To address the hypothesis that altered relative expression of steroid receptor coactivators and corepressors could underlie altered tamoxifen sensitivity in human breast tumours, and since we previously showed that the relative expression of two coactivators (SRA and AIB1) to a corepressor (ROA) is altered in OR+ breast tumours compared their adjacent normal breast tissue, we chose these coregulators to study. They were measured by RT–PCR in the above tumour cohorts. SRA, AIB1, and ROA mRNAs were detectable in most samples, even though their level of expression differed from one sample to another. Consistent with our previous results (Leygue *et al*, 1999b), an additional fragment, migrating at an apparent size of 459 bp was also observed in most tumours when using SRA specific primers. Sequencing analysis revealed that this band corresponded to a variant form of SRA (referred to as SRA-Del) deleted in 203 bp between positions 155 and 357 (positions given correspond to Genbank accession number AF092038). There were no significant differences between the tamoxifen sensitive and the *de novo* tamoxifen resistant breast cancers in the relative expression of any of the coactivators to corepressor RNA, or in the relative expression of SRA/AIB1 RNA, or in expression of any of these coregulator RNAs relative to ORα or total ORβ RNA expression. As well, there was no significant difference in the relative expression of variant SRA/full-length SRA between the groups either.

Tumour characteristics No statistically significant differences were found between the tamoxifen sensitive and tamoxifen resistant cohorts in any of the tumour characteristics described in the Materials and Methods section except for PR. PR levels were statistically significantly different (P=0.044) between the two groups

using a Mann–Whitney rank sum test (two sided). PR levels were higher (median PR was 32 fmol mg^{-1} protein; range 8–216 fmol mg^{-1} protein) in the tamoxifen 'sensitive' group compared to the tamoxifen '*de novo* resistant' group (median PR was 14 fmol mg^{-1} protein; range 4–288 fmol mg^{-1} protein). This was a consistent finding in both selected cohorts (that used for immunohistochemistry and that used for the RNA study), and provides strong support for differences in oestrogen signalling pathways in these two groups since PR is a marker of OR signal transduction (Horwitz *et al*, 1975; Osborne, 1998a).

DISCUSSION

We and others have shown that the relative expression of ORα and ORβ is significantly altered during breast tumourigenesis (Leygue *et al*, 1998; Roger *et al*, 2001), and a similar mechanism has been proposed to underlie tamoxifen resistance in breast cancers (Paech *et al*, 1997). The current study shows no significant differences in expression of full-length ORβ (ORβ1) between tamoxifen sensitive and resistant tumours. Interestingly, in this small cohort of tumours when total ORβ expression was examined, there were significantly more high total ORβ expressors in the tamoxifen 'sensitive' compared to the 'resistant' group. The data suggest the possibility that increased and altered ORβ isoform protein expression may have a role in *de novo* tamoxifen resistance, or at least together with other parameters may provide better markers of endocrine sensitivity. The increased expression of ORβ proteins in the tamoxifen sensitive group is also consistent with recently published data where patients with ORβ positive tumours (determined using an antibody to an N-terminal epitope of the ORβ protein, and defined as nuclear staining in >10% of cancer cells) had a significantly better overall survival than patients with ORβ negative tumours while receiving adjuvant tamoxifen therapy (Mann *et al*, 2001). Both these latter data and those presented currently in this manuscript are in contrast to data showing increased ORβ RNA expression in tamoxifen resistant tumours versus tamoxifen sensitive tumours previously published (Speirs *et al*, 1999). Together these studies suggest that the ORβ status and the nature of ORβ isoforms together with ORα status in human breast cancers may be important biomarkers of endocrine sensitivity, and warrants further study, in larger, prospectively gathered cohorts. The association of increased ORβ isoform expression with tamoxifen sensitivity, suggests a possible mechanistic role, and one possible mechanism may be suggested by several publications which have shown that ORβ isoforms have a modulatory effect on ORα, both in normal tissues (Weihua *et al*, 2000) as well as in cell culture models (Ogawa *et al*, 1998; Hall and McDonnell, 1999).

The potential difference between tamoxifen sensitive and resistant groups with respect to ORβ-like proteins, was not correlated with differences in the relative expression of full-length ORβ and two known variants ORβ2 and ORβ5 at the RNA level between the tamoxifen 'sensitive' versus the tamoxifen 'resistant' groups, however. This may be due to differential regulation of protein versus RNA level or the likelihood that there are other potential ORβ isoforms (known and unknown) expressed in breast tissues in addition to ORβ1, ORβ2 and ORβ5 (Lu *et al*, 1998; Fuqua *et al*, 1999), whose cognate proteins would be detected by the antibody but not measured in the triple primer RT–PCR assay.

Another mechanism for differential tamoxifen sensitivity in OR+ breast tumours could be altered coregulator expression. Although the relative expression of OR coregulators SRA, AIB1 and ROA is altered between normal breast and OR+ breast tumours, there were no significant differences in the ratios of any of the coactivators/corepressors or any of the ratios of these coregulators to ORα RNA levels between primary breast tumours from patients who were later found to be disease free (sensitive) or have disease progression (resistant) while on adjuvant tamoxifen treatment. These data suggest that altered relative expression of these coregulators is unlikely to be a marker of tamoxifen sensitivity in OR+, node negative, primary breast tumours, and unlikely to have a functional role in *de novo* tamoxifen resistance. Although SRA is functional as an RNA molecule, ROA and AIB1 are functional as proteins. Furthermore, other factors can affect protein activity for example phosphorylation in the case of AIB1 (Mora and Brown, 2000) or sequestration by other proteins such as prothymosin-alpha in the case of ROA (Martini *et al*, 2000). Our studies do not exclude differences at the protein and/or activity levels of ROA and AIB1 being involved in *de novo* tamoxifen resistance, nor do they exclude altered expression of these factors having a role in acquired tamoxifen resistance (Lavinsky *et al*, 1998). Altogether, there is little evidence for altered coregulators expression in breast tumours that are *de novo* tamoxifen resistant. However, our data provide preliminary evidence that the expression of ORβ protein isoforms may differ in primary tumours of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy. As well our data support distinct differences in the OR signalling pathways between these two groups of patients since the expression of a known oestrogen responsive gene PR is significantly different between the two groups, the precise mechanisms underlying these differences remain to be elucidated.

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Identification of new human coding steroid receptor RNA activator isoforms☆

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Abstract

SRA is a steroid receptor co-activator which acts as a functional RNA and is classified as belonging to the growing family of functional non-coding RNAs. None of the different SRA transcripts described to date encode a detectable SRA protein following in vitro and in vivo translation experiments. We have identified three new SRA-RNA isoforms differing mainly from the originally cloned SRA by an extended 5′ extremity. These long SRA isoforms, able to encode a stable protein in vitro, led to the production in vivo of a nuclear protein when transfected into the MCF-7 human breast cancer cell line. Reverse-transcription polymerase chain reaction and Western blot analysis of RNA and protein extracts from different breast cancer cell lines confirmed the presence of endogenous coding SRA isoforms and their corresponding proteins. Our results demonstrate that full-length SRA-RNAs likely to encode stable proteins are widely expressed in breast cancer cell lines.

Keywords: SRA; Steroid receptor co-activator; Normal and tumor human breast tissue; Polymorphism; Tumorigenesis; PCR

Endogenous steroid hormones such as estrogen, progesterone, and androgen regulate the growth and the development of several organs and tissues including brain, bones, and reproductive organs such as uterus, testis, and breast. Steroid hormone actions are mainly mediated through specific receptors that belong to the steroid/thyroid/retinoic acid receptor super-family and act as ligand-dependent transcription factors [1–3]. The transcription of target genes by hormone-liganded receptors depends upon interactions between these receptors and several members of a complex co-regulator population (see [2] and references herein). Among co-regulators, co-activators are proteins defined as enhancing hormone induced transactivation without altering basal transcriptional activity and as reversing squelching between different receptors when overexpressed [3]. To an already long list of nuclear receptor co-activators, Lanz et al. [4] recently added SRA, a steroid receptor specific activator that differs from other co-activators in two main features. First, SRA co-activates steroid receptors as an RNA and not as a protein. These authors were unsuccessful in trying to generate in vitro or in vivo stable SRA protein but demonstrated that SRA-RNA existed in a ribonucleoprotein complex activating steroid receptor induced transcription in the absence of a translated SRA protein [4]. Second, as opposed to most positive co-regulators that interact with and co-activate both class I and class II nuclear receptors, SRA appears to be specific for steroid receptors. SRA expression is modified during breast tumorigenesis and breast tumor progression and we have suggested that this co-activator could be involved in the molecular mechanisms underlying these events [5,6]. More recently, it has been shown that antisense oligonucleotides can be used to decrease endogenous SRA-RNA [7] and that this RNA interacts with other proteins such as Sharp and RNA-binding DEAD-box p72/p68 proteins to modulate steroid

☆ *Abbreviations:* SRA, steroid receptor RNA activator; ER, estrogen receptor; PR, progesterone receptor.

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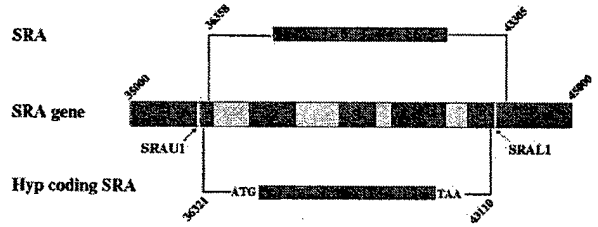

Fig. 1. Schematic representation of SRA–mRNA and gene structure. Human SRA isoforms identified to date differ slightly in their 5′ and 3′ terminal regions but a common nucleotide alignment sequence can be generated. The only SRA sequence entered in GenBank (AF092038) is fully contained within 10,000 bp of Bac 5 genomic sequence (AC005214). Dark gray boxes correspond to exons numbered appropriately. Numbers indicate positions relative to this latter sequence. Hyp coding SRA: hypothetical coding SRA sequence found using Gene Finder. Positions of SRAU1 and SRAL1 primers are indicated by arrows. Light gray boxes are introns.

receptor activity [8,9]. Overall, SRA is now considered as a member of the expanding family of functional non-coding RNAs [10].

The first published sequence for SRA (GenBank AF092038) was fully contained in a genomic sequence of chromosome 5 (GenBank AC005214), within five separated exon-like regions (Fig. 1). Within this latter clone we have found, using Gene finder (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html), the sequence of a hypothetical mRNA which could encode a 236 amino-acid protein that corresponds exactly to the first published SRA sequence, except for an additional 37 nucleotides in the 5′ region (Fig. 1). This new 5′ region now contains an AUG start codon encoding the first methionine of a novel, putative 236 amino-acid SRA protein in contrast to the previously predicted 162 amino-acids which were unstable. We have investigated the expression of this hypothetical mRNA and its corresponding protein in breast cancer cell lines.

Materials and methods

Human breast tissues and cell lines

Breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10AT1, MCF10A1, MCF10AT3B, ZR-75, T47D, T5, MCF7, and HBL100) were grown, harvested, and cell pellets were stored at −70 °C, as previously described [11]. Total RNA and DNA were extracted from frozen normal breast tissue sections (obtained from the Manitoba Breast Tissue Bank) and cell pellets using Trizol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions [12]. Total proteins were extracted from frozen cell pellets as previously described [13].

Primers and RT-PCR conditions

Detection and cloning of the hypothetical coding SRA-RNAs. Primers used consisted of SRAU1 primer (5′-TCCTTTGGTGCC TTGTGAC-3′; sense; positions 36,132–36,150, GenBank Accession No. AC005214) and SRAL1 primer (5′-AGTCTGGGGAACCGAGG AT-3′; antisense; positions 43,128–43,110, GenBank Accession No. AC005214). One microgram of total RNA was reverse transcribed in a final volume of 25 µl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers as previously described [13,14]. The PCR Optimizer Kit (Invitrogen, Carlsbad, CA) was used to set up optimal RT-PCR amplification conditions. One microliter of RT mixture was amplified in a final volume of 30 µl, in the presence of 60 mM Tris–HCl (pH 8.5), 15 mM $[NH_4]_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer, and 1 U Taq DNA polymerase (Gibco-BRL). Each PCR consisted of a 5 min pre-incubation step at 94 °C followed by 30 cycles of amplification (30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C). PCR products were sub-cloned using TOPO TA cloning kit (for sequencing and in vitro translation experiments) and pcDNA3.1/V5-His TOPO TA expression kit (for stable expression) according to the manufacturer's instruction (Invitrogen, Carlsbad, CA) and sequenced as previously described [14].

Detection of SRA isoform 3. Primers used consisted of SRAU2 primer (5′-GGGCCTCCACCTCCTTCAAGTA-3′; sense; positions 41664–41685, GenBank Accession No. AC005214), SRAL2 primer (5′-GCAGTCTTCCAATGCCTG-3′; antisense; positions 41813–41796, GenBank Accession No. AC005214), and SRAL-**GTCG** primer (5′-C ACATCCTCCATCA**GTCG**-3′; antisense; positions 41780–41767 which is specific for SRA isoform 3, GenBank Accession No. AC005214 plus **GTCG** sequence). Radioactive PCR amplifications were performed in the presence of [α-^{32}P]dCTP and SRAU2/SRAL2 or SRAU2/SRAL-**GTCG** primers and PCR products were separated on poly-acrylamide gels as previously described [15]. Following electrophoresis, the gels were dried and exposed 30 min to a Molecular ImagerTM-FX Imaging screen (Bio-Rad, Hercules, CA). Exposed screen was then scanned using a Molecular ImagerTM-FX (Bio-Rad, Hercules, CA). As positive control, we amplified 10 ng of sequenced plasmids previously shown to correspond to SRAcod1, SRAcod2, and SRAcod3 inserted.

In vitro synthesis of SRA protein

[^{35}S]Methionine-labeled SRA proteins were generated in vitro using Reticulocyte Lysate coupled transcription/translation reactions by the TnT System (Promega, Madison, WI) according to the manufacturer's instructions using expression plasmids corresponding to SRAcod1, SRAcod2, or SRAcod3 inserts. Lysates were then subjected to SDS–PAGE, after which gels were dried, and [^{35}S]methionine-labeled protein bands were visualized by exposing overnight to a Molecular Imager-FX Imaging screen (Bio-Rad, Hercules, CA) and subsequently scanned using a Molecular Imager-FX (Bio-Rad, Hercules, CA).

Stable transfection and immunofluorescence. MCF7-SRA1 and MCF7-SRAN cells were engineered by stably transfecting MCF-7 breast cancer cells with pcDNA3.1/V5-His TOPO TA vector containing the full SRA1 coding sequence (between primers SRAU1 and SRA-L3, see Fig. 2) either in frame with a C-terminal V5-His Tag (SRA1) or in an inverse orientation (SRAN), using Lipofectamine reagent according to the manufacturer's protocol (Life Technologies, Gaithersburg, MD). To insure the retention of the transgene, transfected cells are maintained in the presence of Geneticin (Gibco-BRL, Grand Island, NY, 500 µg/ml). MCF7-SRA1 and MCF7-SRAN cells grown on 4-well slides were fixed in freshly prepared 3.7% paraformaldehyde (30 min at 37 °C). Following fixation, slides were blocked in 1% FBS–PBS (overnight, 4 °C) and incubated with anti-V5 (Invitrogen, Carlsbad, CA, 1:750 dilution) primary mouse antiserum (1 h, room temperature). Slides were thoroughly washed in PBS followed by addition of the Cy3-conjugated goat anti-mouse secondary antiserum (Jackson Immuno-Research, 1 h, 37 °C, 1:10,000 dilution). Slides were counterstained with Hoechst (1 mg/ml, 30 min), washed extensively with PBS, mounted in FluorSave mounting reagent (Calbiochem), and visualized using an E1000 Nikon microscope (UV-2A or G-2A filters) with epifluorescence illumination and a DXM 1200 Nikon camera. All images were processed using Act-1 (Nikon) and Adobe Photoshop software.

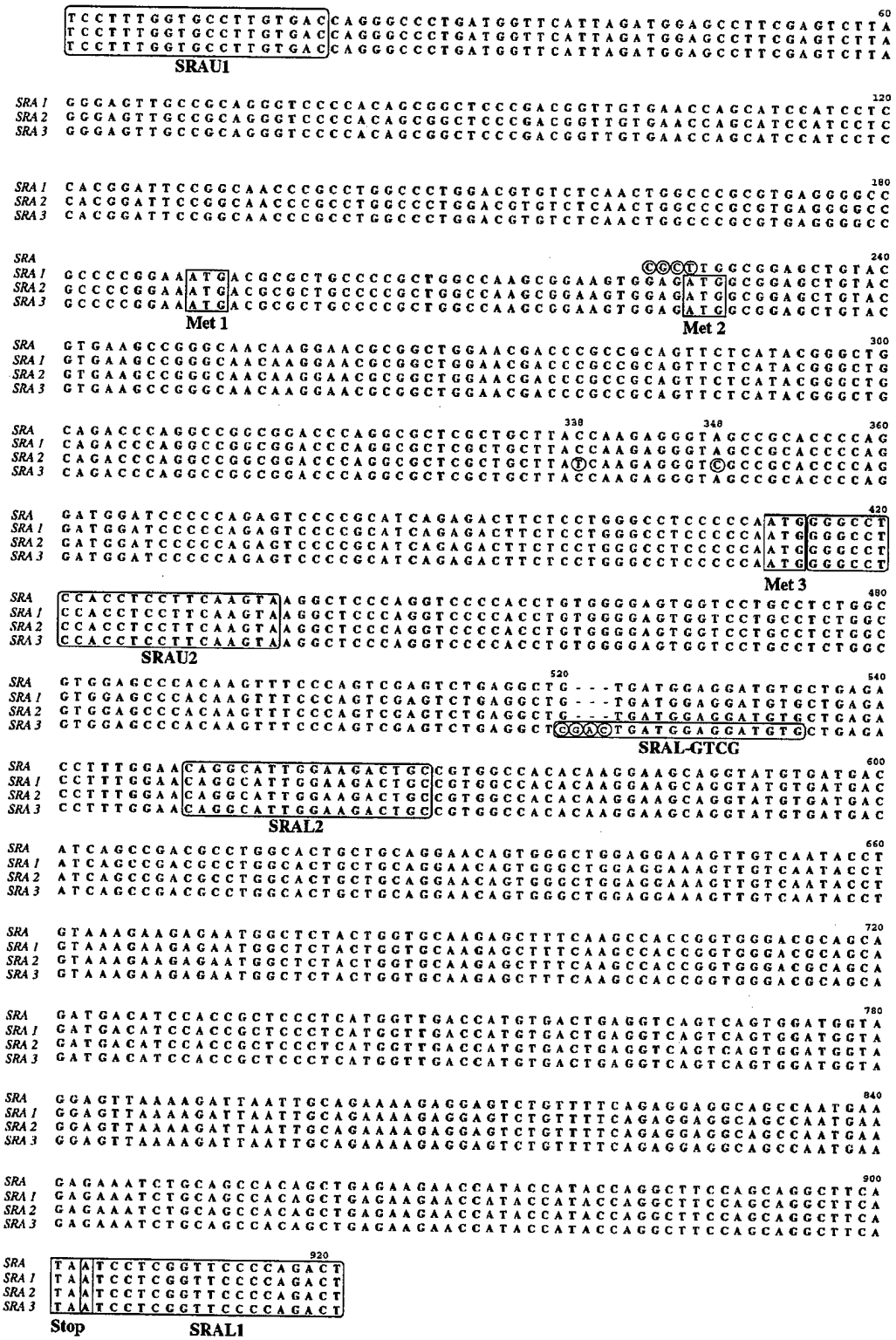

Fig. 2. Alignment of SRA isoforms and primer positions. Previously (4) cloned SRA sequence (AF092038) is aligned with new SRA isoforms: SRA1 (AF293024), SRA2 (AF293025), and SRA3 (AF293026). Differences between sequences are circled. Positions of two new putative starting ATG codons are indicated (Met 1 and Met 2), together with that of the initially predicted starting codon (Met 3, [4]) and the common stop codon. The positions of SRAU1, SRAU2, SRAL-GTCG, SRAL2, and SRAL1 primers are also depicted.

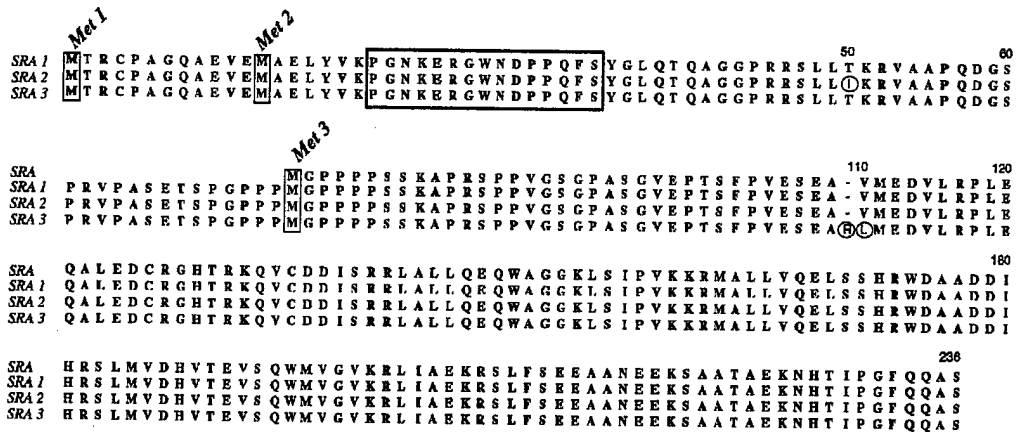

Fig. 3. Alignment of proteins putatively encoded by SRA isoforms. SRA, SRA1, SRA2, and SRA3: proteins putatively encoded by open reading frames contained in AF092038, AF293024, AF293025, and AF293026, respectively. Positions of two putative first methionine (Met 1 and Met 2), together with initially predicted first methione (Met 3, [4]), are indicated. Differences between sequences are circled. Fifteen amino acids highlighted by a box show recognition site for antibody generated against SRA.

Western blot. Protein extracts were separated on SDS–polyacrylamide gels and transferred onto nitrocellulose membranes as previously described [13]. An affinity purified rabbit polyclonal antibody (anti-SRA) raised against the peptide PGNKERGWNDPPQFS (see Fig. 3) was obtained from ResGen (Invitrogen, Carlsbad, CA). Immunodetection of SRA protein was performed using anti-SRA antibody as a primary antibody and a goat-anti-rabbit-HRP conjugated antibody as a secondary antibody as previously described [13]. Antibody neutralization experiments were performed by pre-incubating SRA antibody with its corresponding peptide (2 h, room temperature).

Results

Detection of hypothetical coding SRA isoforms in normal breast tissue

To determine if the hypothetical protein encoded by SRA–mRNA was expressed in vivo, primers were designed corresponding to sequences upstream of the putative first AUG codon (SRAU1) and downstream of the putative stop codon (SRAL1). Total RNA was extracted from two normal breast tissue samples. Reverse transcription and PCR amplification were performed as described in Materials and methods. Using these primers, we obtained the predicted 920-bp PCR product (data not shown). Cloning and sequencing of this fragment revealed that it essentially corresponded to the hypothetical SRA coding sequence (Fig. 2). Interestingly, three different SRA–cDNAs were identified: SRA isoform 1 (GenBank AF293024), SRA isoform 2 (GenBank AF293025), and SRA isoform 3 (GenBank AF293026). SRA isoform 1 contained the full coding hypothetical SRA, whereas SRA isoform 2 contained two point mutations at positions 338 (C → T) and 348 (A → C), and SRA isoform 3 contained a point mutation followed by an insertion of three nucleotides at position 520 (G → CGAC). All these sequences contained a potential open reading frame able to encode two 236 aa and one 237 aa protein for SRA isoforms 1, 2, and 3, respectively (Fig. 3).

In vitro translation of three new SRA isoforms: SRA isoforms 1, 2, and 3

Previously cloned SRA–cDNAs, in which only the third ATG codon (encoding Met 3, Figs. 2 and 3) was present, were unable to support detectable protein synthesis in vitro [4]. To determine if the three new SRA–cDNAs isolated in our laboratory could be translated in vitro, expression vectors containing SRA isoform 1, 2, and 3 sequences downstream of a T7 polymerase promoter were used in a TnT coupled Reticulocyte Lysate system as described in Materials and methods. The three different SRA isoforms encoded stable SRA proteins were produced under these conditions (Fig. 4). Sur-

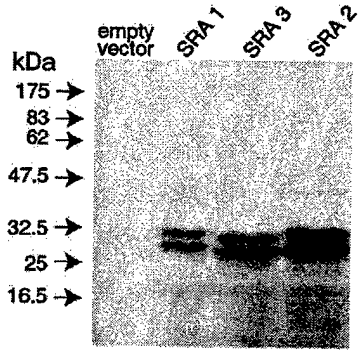

Fig. 4. In vitro translation of SRA isoform 1, 2, and 3 cDNAs. In vitro transcription/translation reactions were performed using SRA isoforms 1, 2, and 3 corresponding expression plasmids and labeled/unlabeled lysates analyzed as described in Materials and methods. Size in kilodalton, corresponding to the prestained marker, broad range (Premixed format, BioLabs), are shown on the left.

prisingly SRA3 encoded protein, even though one amino acid longer is migrating slightly faster than its SRA1 and SRA2 counterparts. It should also be stressed that two bands were observed for each construction, consistent with the possible use of two different initiating methionine codons (Figs. 2 and 3, Met 1 and Met 2). The observed molecular masses (31 and 30 kDa for SRA isoforms 1–2 and 3, respectively) were slightly higher than those predicted (25.7 and 25.8 kDa for SRA isoforms 1–2 and 3, respectively).

Detection of SRA proteins in vivo by transfection of long SRA isoforms

To check whether the newly isolated long SRA isoforms could be translated in vivo, MCF-7 breast cancer cells were stably transfected with expression vectors encoding SRA1, SRA2, or SRA3 protein in frame with a C-terminal V5-His tag. The presence of SRA-tagged proteins in SRA transfected cells only was confirmed by Western blot performed using anti-V5 antibodies (data not shown). As shown Fig. 5A, SRA protein localizes in both the cytoplasm and the nucleus of SRA transfected cells whereas no signal was seen in control cells (MCF7-SRAN cells) transfected with an antisense SRA sequence (Fig. 5B).

Detection of endogenous coding SRA isoforms in breast epithelial cell lines

To determine if the long coding SRA sequences are expressed in breast cancer cells, total RNA was extracted from different breast cancer cell lines and amplified as described. The predicted PCR product corresponding to the hypothetical coding SRAs was observed in all cell lines (Fig. 6A), confirming these transcripts are expressed in tumorigenic and non-tumorigenic human breast epithelial cells. Blast searches of human EST databases (http://www.ncbi.nlm.nih.gov/blast/) revealed that the specific sequence of SRA isoform 3 (i.e., G → CGAC at position 520) had already been cloned by others (GenBank Accession Nos.: AW954396, AW957456, AW 630779, AA305793, AA410852, and AA353911). This confirmed our own data resulting from independent RT-PCRs, cloning, and sequencing and underlined that the SRA isoform 3 was not the result of a technical artifact. Therefore, it was of interest to investigate the expression of this isoform in breast cancer cells. PCR primers were designed to specifically amplify a fragment overlapping the putative insertion region (SRAU2, SRAL2) or to specifically anneal with the inserted sequence (SRAL-**GTCG**) (see Fig. 2 for primer positions). PCR products amplified using the former set of primers (SRAU2, SRAL2) were expected to migrate at an apparent size of 150 and 153 bp for SRA isoform 1–2 and 3 cDNAs, respectively. Using the latter set of primers (SRAU2, SRAL-**GTGC**), a PCR product 117-bp long was expected only in samples expressing SRA isoform 3 mRNA. Results obtained using SRAU2/SRAL2 and SRAU2/SRAL-**GTGC** are shown Figs. 6B and C, respectively. Interestingly, some cell lines expressed only the SRA isoform 3 specific fragment (MDA-MB-231, MCF-7) whereas others expressed both SRA isoforms 1–2 and 3 (T47D, T5).

DNA extracted from these cell lines was also amplified using SRAU2 and SRAL2. As shown in Fig. 6D a

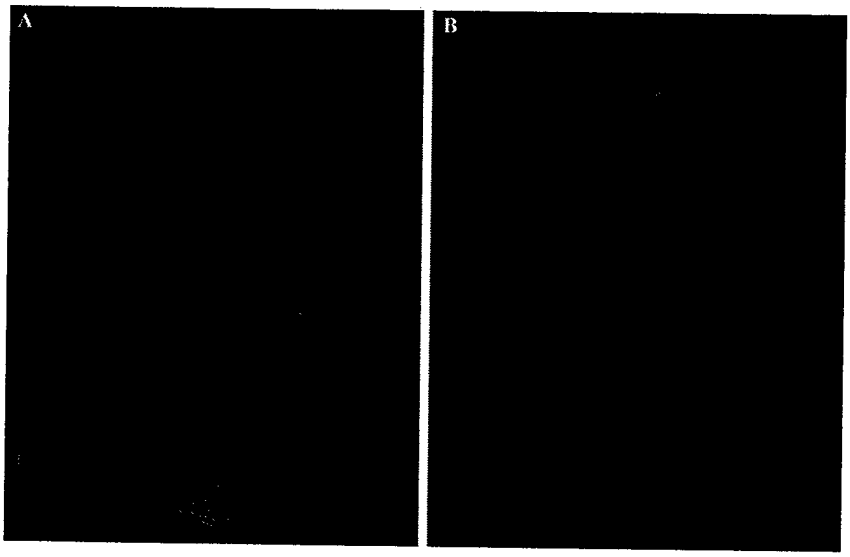

Fig. 5. Detection in vivo of SRA proteins transfected in MCF-7 cell line. MCF7-SRA1 (A) and MCF7-SRAN (B) cells were engineered by stably transfecting MCF-7 breast cancer cells with C-terminal V5-tagged SRA1 sequence or SRA antisense sequence (SRAN). V5 epitope was visualized by immunofluorescence (red signal) and nuclei were counterstained using Hoechst (blue signal) as described in Materials and methods.

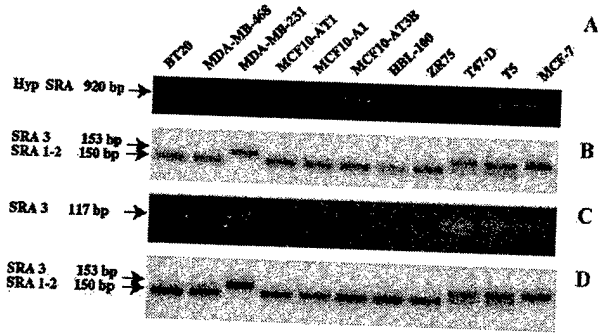

Fig. 6. Detection of SRA isoforms in breast epithelial cell lines. Total RNA was extracted from breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10AT1, MCF10A1, MCF10AT3B, HBL-100, ZR-75, T47D, T5, and MCF-7), reverse transcribed, and PCR-amplified using SRAU1/SRAL1 (A), SRAU2/SRAL2 (B), or SRAU2/SRAL-GTCG (C) primers and PCR products were separated on agarose (A, C) or acrylamide (B) gels, as described in Materials and methods. (D) Genomic DNA corresponding to these cell lines was amplified using SRAU2/SRAL2 and PCR products were separated on acrylamide gel as described in Materials and methods.

perfect correlation existed between SRA isoform expression and DNA sequence, suggesting the possible existence of a genetic polymorphism and therefore of at least two alleles of the SRA gene.

Detection of endogenous SRA proteins in breast cancer cells

A rabbit polyclonal anti-SRA antibody was generated and Western blots were performed on breast cancer cells

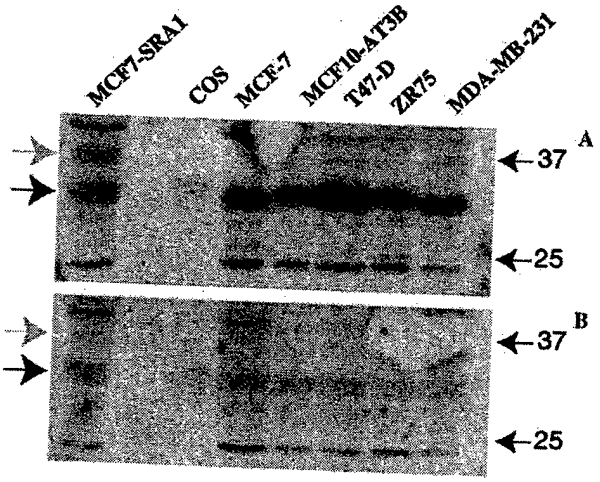

Fig. 7. Western blot detection of endogenous SRA proteins in breast cancer cell lines. Total proteins were extracted from different breast cancer cell lines (MCF-7, MCF10-AT3B, T47-D, ZR75, and MDA-MB-231), separated on SDS–PAGE, and transferred on nitrocellulose membrane. Western blot detection of SRA protein was performed using SRA antibody (A) or SRA antibody neutralized with the corresponding peptide (B), as described in Materials and methods. MCF-SRA1: MCF-7 transfected with SRA1-V5-tagged construction. Cos: Cos cells. Gray arrow: SRA-V5-tagged protein localization, black arrow: endogenous SRA localization.

(Fig. 7) as described in the Materials and methods. A clean signal, absent when the antibody is pre-incubated with the corresponding peptide, is observed at the expected position (~30 kDa) in different breast cancer cell lines. The specificity of the signal observed is further confirmed by the fastest apparent SRA protein migration (SRA3 protein, see Fig. 4) in MCF-7 and MDA-MB-231 cells, shown by RT-PCR to express only SRA3 isoforms.

Discussion

We have identified three new SRA isoforms, longer in their 5′ extremity than those previously described. Database searches revealed that although many partial SRA-like sequences isolated from various normal and tumor tissues have been entered in the human EST sequence database, only a few appear to correspond to these full-length hypothetical protein coding SRA-RNAs. This suggests an overall low expression of these long isoforms or a relative tissue specificity. Interestingly, when investigating SRA-RNA expression by Northern blot Lanz et al. reported the existence of several different sized transcripts expressed in a tissue and cell specific manner. In particular, a 1400 base long SRA-RNA, large enough to contain the minimum 920 bases of our new long SRA isoforms, was strongly expressed in muscle but not in kidney. Such data suggest that the expression of the long hypothetical coding SRA isoforms we described in this study may be tissue specific.

We observed that either one or two SRA isoforms can be expressed in breast epithelial cells. In the situation where two isoforms are expressed at the same time, both alleles are actively expressed. It should be noted that our PCR assays (using SRAU2/SRAL2 and SRAU2/SRAL-GTCG) do not establish whether SRA isoforms 1 and/or 2 are expressed. These assays establish only whether or not SRA isoform 3 is expressed, alone or together with one of the other isoforms (SRA isoform 1 or 2). We found that in breast cancer cells the pattern of SRA isoform expression was directly related to their detection within the genomic DNA. These data suggest the existence of genetic polymorphisms within the SRA gene.

All non-coding SRA isoforms identified by Lanz et al. [4] shared a core sequence starting in exon 2 and stopping in exon 5. This core sequence has been shown to be necessary and sufficient for these RNAs to act as co-activators of steroid receptors. As the longer isoforms we describe here contain this core sequence, it is expected that these latter RNAs could also modify steroid receptor activity as do their shorter previously described counterparts.

Our data are the first to report the detection of an endogeneous SRA protein and to show that naturally expressed human SRA-RNAs can generate a cDNA encoding a detectable SRA protein in vitro and in vivo.

Lanz et al. [4], who reported the identification of three different SRA isoforms, concluded that none of their cloned SRA–cDNA sequences (i.e., predicted to encode a 162 aa protein) could encode a detectable translation product. Interestingly, when engineered to form a fusion protein, e.g., with GST, Gal4 or HSV-thymidine kinase initiation sequences attached to the N-terminal region, the appropriate fusion SRA-like product was detected. Taken together, these data suggest that the 5′ extremity of their cloned SRA was missing a functional initiating methionine codon or that the extra 74 N-terminal amino acids predicted in our hypothetical coding SRA–mRNAs are required for the stable expression of the resulting protein. This is reflected by the production of two different sized proteins in vitro and in vivo from each of our isoforms, presumably because of a choice in initiation start sites. Interestingly, during the preparation of this manuscript, Karashiwa et al. [16] reported the translation in vitro of a rat SRA related molecule (SRAP). When comparing SRAP protein sequence with our SRA1 protein (entered into GenBank in 2000), these authors observed a 78% conservation in amino acid sequence identity in the 146 amino-acid C-terminal region of SRA1 protein. The putative SRAP in the rat is much smaller (16 kDa) than the human SRA proteins we describe here. Karashiwa et al. were able to express in vivo a rat SRAP protein when fused with the C-terminal extremity of GST or GFP. However, these authors have still to prove the stable expression in vivo of an SRAP protein from its own naturally occurring initiation AUG codon, as well an endogenous rat SRAP protein. Of considerable interest is the function described for this SRAP protein. Kawashima et al. reported that SRAP directly interacted with the androgen receptor (AR) and the glucocorticoid receptor (GR) to increase the transcriptional activity of these receptors. As the human SRA proteins share a strong sequence homology with the rat SRAP in their C-terminal domain, we hypothesize that human SRA protein could also interact with and modify the activity of steroid receptors, as do their RNAs. The nuclear localization of SRA protein suggests the possibility of such an interaction. Further experiments are needed to clarify the exact function of these new long SRA isoforms and their encoded proteins. However, if our latter hypothesis is confirmed, SRA might become the first molecule to be active in the same signalling pathway both at the RNA and at the protein levels.

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